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MIDAZOLAM NO ESTRESSE POR CONTENÇÃO EM AVES SILVESTRES

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LAURA GARCIA VILA

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"O saber a gente aprende com os mestres e com os livros, a sabedoria, se aprende é com a vida e com os humildes."

Cora Coralina

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RESUMO

O estresse produzido pela contenção em aves silvestres pode resultar em graves complicações. A procura por alternativas que minimizem tais riscos está plenamente justificada, sendo uma delas, a sedação. No presente estudo a sedação com midazolam foi testada em duas espécies de aves silvestres. Vinte e uma araras canindé foram randomizadas e divididas em dois grupos, um recebeu solução salina (grupo controle) e o outro aproximadamente 7,5 mg/kg de midazolam (grupo midazolam) via spray intranasal, em dois períodos de tempo diferentes, separados por dois meses. Catorze emas foram submetidas a um protocolo semelhante, porém a dose do midazolam foi 1 mg/kg,via intramuscular. Após 10 min do momento da aplicação do sedativo/salina foi feita a avaliação física (frequência cardíaca, frequência respiratória e temperatura) e a colheitado sangue por punção venosa. Foi realizada hemogasometria (somente nas araras), hematologia e bioquímica das amostras sanguíneas. Nas araras, a aplicação do midazolam produziu sedação, valores mais baixos para os parâmetros de avaliação física e concentrações significativamente ($p<0.05$) mais baixas de lactato, excesso de base e cloro enquanto que os valores de pCO_2, $ctCO_2$ e HCO_3 foram mais elevados em comparação ao grupo controle. Nas emas, a única diferença significativa observada foi na frequência respiratória, sendo maior no grupo midazolam. Nas araras, o midazolam atenuou a acidose metabólica derivada da contenção, apresentando efeito na redução do estresse, mostrando-se uma alternativa segura e efetiva. A resposta ao midazolam nas emas variou em função do indivíduo,sem efeito consistente na redução do estresse.

Palavras-chave:acidose metabólica,*Ara ararauna*, bioquímica clínica,hematologia, hemogasometria,miopatia por captura,*Rhea americana*.

ABSTRACT

MIDAZOLAM ON RESTRAINT INDUCED STRESS IN WILD BIRDS

Restraint induced stress in wild birds can be life threatening. Research for alternatives that minimize those risks iswell justified, sedation beinga suitable option. In the present study midazolam sedation was tested in two species of wild birds. Twenty-one blue-and-yellow macaws were randomly assigned in two groups, one receiving saline solution (control group) and the other approximately 7.5 mg/kg of midazolam (midazolam group) via an intranasal spray, in two different moments with a washout period of two months. Fourteen greater rheas were submitted to a similar protocol; however, the midazolam dosage was 1 mg/kg via an intramuscular route. After 10 min following sedative/saline application, physical exam (heart rate, respiratory rate and temperature) and venous blood collectionwere performed. Blood samples were processed for hemogasometry (only with macaws), hematology and biochemistry analyses. In macaws, midazolam application produced sedation; lower values for physical exam parameters and significantly ($p<0.05$) lower lactate, base excess and chloride concentrations, while pCO_2, $ctCO_2$ and HCO_3 values were higher when compared with control group. In rheas, the only significant difference was the respiratory rate, being higher in the midazolam group. In macaws midazolam attenuated metabolic acidosis derived from restraint, showing a positive effect on stress reduction, proving an effective and safe alternative. In response to midazolam rheas showed inconstant reaction depending among individuals, with no reliable effect on stress restraint reduction.

Keywords:*Ara ararauna*,metabolic acidosis,capture myopathy, clinical biochemistry, hematology,hemogasometry,*Rhea americana*.

CAPITULO 1 - CONSIDERAÇÕES INICIAIS

1 Introdução

Aarara canindé (*Ara ararauna*, Linnaeus, 1758) e a ema (*Rhea americana*, Linnaeus, 1758) são espécies de aves representativas do Cerrado Brasileiro. A distribuição geográfica de ambas espécies é ampla.Asararas canindé são observadas no sul da América Centrale em vários países da América do Sul, regiões de clima tropical, ocupando os biomas Amazônia, Cerrado e Pantanal. As emas habitam o nordeste e o sudeste do Brasil, leste da Bolívia, Uruguai e Paraguai e o nordeste e leste da Argentina[1,2,3].Alterações nas populações de aves silvestres geram impactos de grande escala nos ecossistemas onde se encontram[4], justificando a preocupação com a preservação de cada uma das espécies.

A arara canindé pertence a ordem Psitaciformes, família Psittacidae[2]. Está classificada como "*Last concern*" na lista vermelha de espécies ameaçadas da União Internacional para a Conservação da Natureza e dos Recursos Naturais (UICN). Porém, a diminuição gradual das suas populações fez com que fosse adicionada no Apendix II da Convenção sobre o Comércio Internacional das Espécies da Fauna e da Flora Selvagens Ameaçadas de Extinção (CITES), pois é uma dasaves mais contrabandeadas do seu genero[5]. Esta é uma espécie muito apreciada como animal de estimação, sendo muito comum em criadouros comerciais, bem como, são frequentes os atendimentos em clínicas veterinárias[6]. No Brasil e em outros países onde a espécie é endêmica, estão sendo feitos trabalhos de reintrodução de animais de procedência diversa que chegam aos centros de triagem[7].

A ema pertence à ordem Struthioniformes, familia Rheidae. É a maior ave do continente sul americano e, frequentemente, é agrupada sob o termo "ratitas" junto a outras espécies de aves não voadoras como avestruz, emu, cassuar e kiwi. Estas espécies são filogeneticamente distantes e sofreram uma evolução convergente em prol da adaptação ao meio terrestre[8]. A ema foi classificada como "*Near threatened*" na lista vermelha da UICN devido a rápida diminuição nas populações da espécie[3]. A crescente diminuição de habitat em decorrência do avanço da agricultura é uma das principais razões que ameaçam a ave[9],juntamente com a caça ilegal e a colheita de ovos[10]. Muitos estudos sobre a situação da conservação das populações silvestres foram realizados na Argentina[9-12], mas no Brasil, as informações sobre o estado e o impacto na espécie da crescente perda de áreas nativas de pastagem,são escassas. A criação em cativeiro é habitual, com objetivo

comercialparaexploração da carne, penas e subprodutos;ou com fins conservacionistas e/ou como parte de programas de reintrodução[11,13].

Tanto nos indivíduos de vida livre como naqueles criados em cativeiro o estresse derivado da captura e contenção pode ser fatal, sobretudo em animais debilitados ou doentes. Tais procedimentos são necessários na clínica, programas de reintrodução, produção e estudos científicos envolvendo esses animais[14-19]. Existem relatos de porcentagem considerável de óbitos decorrentes da manipulação[20-22], que nem sempre ocorrem no momento da contenção, podendo ser decorrentes de lesões secundárias que afetam o desempenho dos animais, tornando-os mais susceptíveis à predação e a problemas cardíacos[23,24]. Uma das mais graves consequências do estresse por contenção é a miopatia por captura, causa comum de óbito durante a manipulação de animais silvestres[20,25].

As informações sobre os valores hematológicos e bioquímicos para as diferentes espécies de aves ainda são escassas, entretanto, são fundamentais na prática clínica para estabelecer diagnóstico eficiente e tratamento rápido,uma vez que muitos destes animais chegam na clínica em estado crítico. A dificuldade em estabelecer diagnóstico precoce é inerente a esse grupo de animais, que por serem silvestres ocultam sinais de doença, que só serão detectados quando em estádios muito avançados de evolução. Esta estratégia é compreensível em situações de vida livre como mecanismo para evitar à atenção de predadores potenciais, mas na clínica, representa uma complicação ou até mesmo a impossibilidade do tratamento[26,27]. O estabelecimento de valores de referência é difícil devido à influência de múltiplos fatores como a região geográfica, a idade ou estilo de vida[28-30]. Além disso, os valores publicados na literatura muitas vezes contemplam números pequenos de animais. Deste ponto de vista, o conhecimento dos valores obtidos pelos exames laboratoriais é de suma importância. No caso concreto das emas, são pouquíssimos os dados publicados referentes e esses valores e comparações com outras espécies de ratitas parecem inapropriadas devido à distância filogenética entre as espécies deste grupo.

Diante do exposto, a procura por técnicas que minimizem o risco no trabalho com espécies silvestres está plenamente justificada. Além disso, a contribuição na geração de informações sobre valores laboratoriais para estas duas espécies é de grande importância para os trabalhosde caracterização e de recuperação, assim como em outras aplicações como na clínica de aves silvestres ou na criação comercial.Com este estudo objetivou-seavaliar os efeitos do midazolam sob o estresse por contenção em araras canindé e emas, considerando as mudanças nos parâmetros físicos, hematológicos e bioquímicos.

2 Revisão de literatura

A miopatia por captura, também conhecida por miopatia por exercício ou rabdomiólise por exercício, entre muitos outros termos, apresenta grande semelhança com outras doenças musculares degenerativas que afetam animais domésticos, assim como a rabdomiólise por exercício que acomete os humanos[31,32]. A sua ocorrência relaciona-se comumente a perseguição, captura, contenção e transporte de animais. Trata-se de uma síndrome de caráter multifatorial que pode também apresentar-se secundariamente a outras afecções. Pode-se dizer que é originada por uma resposta ao estresse, sendo os animais silvestres muito mais suscetíveis que os domésticos[32]. A fisiopatologia da miopatia por captura pode ser considerada idêntica à do choque, ou seja, um ciclo vicioso associado à hipoperfusão tecidual e hipóxia[31]. Diferentes autores têm proposto vários sistemas de classificação para a miopatia por captura dependendo dos sinais, lesões e tempo de apresentação[32]. Mas, em linhas gerais, os sinais caracterizam-se por ataxia, dispneia, diferentes graus de miopatia que podem levar até a paralisia e mioglobinúria, assim como falha cardíaca e, finalmente, óbito. Esses sinais podem se manifestar em diferentes graus, assim como aparecer imediatamente após a captura, horas ou dias após o evento estressante[33]. Em todos os casos, as enzimas séricas musculares apresentam atividade elevada. As lesões histológicas confirmatórias são a degeneração muscular aguda ou subaguda, afetando tanto o músculo cardíaco como o esquelético[23] e, habitualmente, o dano tubular, muitas vezes associado a mioglobina tubular[34]. Também podem ocorrer áreas de necrose nos túbulos renais, cérebro, fígado, pâncreas, coração, baço, linfonodos e glândulas adrenais[32].

Existem vários fatores predisponentes, como a maior suscetibilidade de determinadas espécies[32]. Nos mamíferos, destacam-se os ungulados silvestres e, estudos recentes, também sugerem alta incidência em mamíferos marinhos encalhados[34,35,36]. As aves com patas compridas, como as emas, estão particularmente predispostas a sofrer miopatia por captura[32,37-39].

Outros fatores que determinam a aparição desta alteração estão relacionados ao ambiente (alta temperatura e umidade), às técnicas de captura que fazem com que o animal se debata em excesso, ao tempo de imobilização prolongado[25] ou aqueles que provocam uma resposta estressante exacerbada, bem como outras doenças concomitantes que debilitam o animal[23]. Animais idosos, muito jovens[23] ou que sofrem deficiências nutricionais também são mais sensíveis à doença[25]. Por último, algumas drogas usadas para a contenção, como os

derivados opióides, que provocam agitação, rigidez muscular, hipoventilação, acidose muscular[25] e liberação de catecolaminas, aumentam o risco de ocorrência de miopatia por captura[32].

A fisiologia da resposta ao estresse gerado pela contenção é mediada pelo sistema límbico, que estimula o hipotálamo e atua, principalmente, por duas vias: o sistema nervoso simpático e o eixo hipotálamo-hipófise-adrenal[31].O sistema nervoso simpático, junto com o estímulo da medula da adrenal que libera catecolaminas, permite uma resposta rápida e em massa, conhecida como "de fuga ou luta"[40]. Esta resposta acontece quando o hipotálamo é ativado subitamente, como nas situações estressantes[31]. A razão entre as diferentes catecolaminas (adrenalina/noradrenalina) liberadas depende da espécie e da idade de cada animal[32]. O efeito da estimulação simpática provoca aumento da frequência cardíaca, do débito cardíaco e da pressão arterial[31]. A irrigação do músculo esquelético, cardíaco, cérebro e pulmões aumenta em detrimento dos órgãos viscerais, rins, tecido conjuntivo e pele, que experimentam vasoconstrição[31,33,40]. Também é produzida contração do baço que se manifesta pelo aumento de volume globular em até 50% e mobilização de leucócitos dos compartimentosmarginais para à circulação (leucocitose com neutrofilia e/ou linfocitose dependendo da espécie)[41]. Nas aves, o baço não tem a função de reservatório de eritrócitos[42]e a resposta ao estresse está relacionada à leucocitose com heterofilia e linfopenia[43].

Há aumento das taxas de metabolismo celular, maior agregação plaquetária, diminuição da motilidade do trato gastrointestinal e relaxamento da bexiga urinária com contração dos esfíncteres[92]. Ocorre aumento da glicólise no músculo e no fígado, pois é liberado o glucagon e inibida a insulina, resultando na elevação da concentração sanguínea de glicose[43].

Na medula adrenal são sintetizadas e liberadas catecolaminas, cuja função, entre outras, é modular a produção de imunoglobulinas pelas células T e promover a proliferação de linfócitos e células natural *killer*. A estimulação simpática também provoca a liberação de peptídeos vasoativos intestinais e substância P nos tecidos[32]. Também se produz um aumento da força muscular e da atividade mental. Ocorre dilatação pupilar, que permite maior eficiência da visão a distância, e aumento da ventilação pulmonar, concomitante à dilatação bronquiolar[32].

Esta resposta é conhecida como a reação de alarme e permite ao animal realizar um esforço físico muito maior do que seria possível em outras situações[31]. O sistema nervoso simpático e a medula adrenal, em situações normais, se encarregam de manter o tônus dos

vasos sanguíneos[31]. Em situação de estresse grave, a exaustão simpática e adrenal podem levar a hipotensão, colapso vascular e morte[31].

A segunda via, a hipotalâmica (eixo hipotálamo-hipófise-adrenal), constitui a resposta hormonal predominante. O hormônio liberador de corticotrofina (CRH), sintetizado no hipotálamo, induzna hipófise a liberação do hormônio adrenocorticotrófico (ACTH)que promove a estimulação do córtex adrenal, ocasionando a liberação de glicocorticóides (principalmente cortisol e corticosterona)[23]. A proporção entre esses, varia dependendo do táxon, sendo a corticosterona predominante em aves e roedores; o cortisol em felinos, ungulados e primatas e ocorrem em proporções equivalentes nos caninos[32].

Os efeitos desses hormônios se manifestam como aumento da concentração plasmática de aminoácidos, glicerol, ácidos graxos e da glicose sanguínea, disponibilizando assim energia e material para a reparação de possíveis danos celulares[19]. A hiperglicemia é causada por glicólise hepática, inibição da captação de glicose pelas células e aumento no catabolismo de lipídios e proteínas[32]. Os glicocorticóides também produzem vasoconstrição, além de desempenharem papel anti-inflamatório por meio da inibição da resposta imunológica específica[32]. No hemograma, seus efeitos se manifestam como leucocitose com neutrofilia, linfopenia e eosinopenia[41], uma vez que causam a lise e marginação de células T, monócitos e eosinófilos e a diminuição da proliferação de células linfóides[31]. Nas aves, os glicocorticóides provocam influxo de heterófilos desde a medula óssea e atenuam a saída destes para outros compartimentos[43]. Contrariamente, os linfócitos aderem ao endotélio e migram para os tecidos[43]. Em resposta aos esteróides, também são liberadas lipocortinas, que atuam limitando a ativação dos leucócitos por meio da regulação da fosfolipase A2, que resulta na diminuição da produção de prostaglandinas, tromboxanos e leucotrienos. A inibição das prostaglandinas está também relacionada com ulcerações gástricas e duodenais[31].

A estimulação da hipófise pela CRH também se manifesta na liberação de beta endorfinas e outros mediadores. Hipoteticamente estes cumprem funções relacionadas a analgesia, para possíveis danos ocasionados pelo estresse, ao aprendizado, as alterações da conduta e a supressão da fome[32].

Outras mudanças hormonais produzidas durante o estresse agudo são a ativação do sistema renina-angiotensina-aldosterona, como consequência da vasoconstrição mediada pelo sistema nervoso simpático e, por causa disso, também se produz um aumento na secreção de vasopressina[32]. Esses mecanismos ajudam a aumentar o volume plasmático (pela retenção de água e sódio) restituindo possíveis perdas por hemorragia ou suor[32]. Também têm função vasoconstritora, assegurando assim a manutenção da pressão arterial[32].

A atividade muscular também é ativada, pois o medo desencadeado pela perseguição e captura é integrado no tálamo, resultando na ativação do córtex motor[32]. A principal forma de energia utilizada pela contração muscular é o ATP, que provém, em primeiro lugar, das reservas musculares de ATP (que são mínimas), do mecanismo da fosfocreatina (que permite a obtenção de ATP, mas se esgota em 10 a 15 segundos) e da glicólise[32]. Esta última é mais eficiente quando ocorre na aerobiose, mas a demanda energética elevada leva a deficiência de oxigênio, ativando assim, a glicólise anaeróbica (energeticamente menos eficiente) que leva à produção de ácido láctico[32]. A acidose metabólica e o aumento da atividade muscular influenciam também na oxigenação e ventilação, pois essas estão relacionadas ao balanço ácido-base e com as concentrações de eletrólitos[32].

Simultaneamente, ativa-se a "bomba muscular" que ocorre pelo aumento da irrigação muscular e a compressão dos vasos na contração do músculo[31]. Assim, o fluxo de sangue é intermitente, pois quando o músculo relaxa, os vasos enchem rapidamente para se esvaziarem imediatamente com a contração[31]. Essa bomba muscular se mantém ativa enquanto o animal corre, mas para se este é imobilizado, do modo que a contenção colabora com o estado isotônico de contração muscular levando a falha na perfusão tecidual[41], menor dissipação de calor e hipóxia[31].

A hipóxia e consequente acidose láctica, a pouca chegada de nutrientes, o aumento na produção de radicais livres[44] e a falha na remoção de produtos de excreção dos tecidos na ausência da bomba muscular, produzem necrose celular em diferentes graus, que pode levar a coagulação intravascular e trombose[31]. Os metabólitos, tais como a mioglobina e potássio, liberados podem causar uma cascata de complicações como falha renal e disritmias cardíacas que muitas vezes acabam em óbito do animal[32]. A deterioração dos capilares pulmonares também pode levar ao edema pulmonar[31]. O aumento da atividade das enzimas musculares no plasma ocorre devido à lise celular nos músculos[32,33,41,45].Adicionalmente, o aumento na produção de calor originado pelo exercício e a taxa metabólica elevada, junto com altas temperaturas ambientais, também podem desencadear excessivo calor local e agravar a necrose tecidual[31].

As mudanças fisiológicas decorrentes do estresse se manifestam em alterações dos parâmetros físicos, hematológicos e bioquímicos que são mensuráveis por meios laboratoriais. Em aves foram detectados incrementos nas frequências respiratória e cardíaca e na temperatura cloacal em resposta à contenção[19,46,47]. Alterações nos gases sanguíneos e o lactato também foram relacionadas com o estresse por captura em passeriformes[48,49,50].

Incrementos nos valores plasmáticos de enzimas musculares de aves capturadas também foram correlacionados com a intensidade e duração do estímulo estressante e a apresentação de problemas decorrentes da captura[51-53]. Mudanças nas concentrações de metabólitos também foram detectadas em emús submetidos a transporte[15]. As respostas hematológicas desencadeadas pelo estresse em aves variam em função da espécie. Por esse motivo, a razão entre heterófilos/linfócitos foi considerada melhor indicador para o estresse, sendo detectável após 30 minutos e até três horas após o estímulo estressante[43,54].

Em decorrência das graves consequências associadas ao estresse por contenção, os esforços devem concentrar-se na minimização do estímulo de estresse. Desse modo, evitar elevadas temperaturas, reduzir barulhos, estímulos visuais[31,32] e o tempo de contenção[16], oxigenar oanimal e trabalhar com uma equipe de pessoal treinado[31] ajudam a diminuir os riscos associados.

Vários estudos indicam relação entre métodos de captura e a aparição de miopatia por caputra[16,23], sendo, portanto, essencial a escolha da técnica. O fato de imediatamente após a captura não imobilizar completamente o animal, permitindo certa movimentação muscular, ajuda à oxigenação dos tecidos diminuindo o risco da miopatia por captura[31]. São preferíveis aquelas técnicas que não usam a perseguição do animal, como por exemplo, as armadilhas[33].

Um método muito usado para evitar o estresse e facilitar a manipulação de aves é a anestesia. Porém, a anestesia geral está associada a riscos como depressão respiratória, cardíaca, arritmias, hipotermia e depressão cardiovascular[55-57]. Recentemente, o uso de sedativos tem sido uma alternativa cada vezmais usada para evitar os efeitos adversos associados à anestesia geral[56,57]. A sedação nas aves proporciona mais segurança para o manipulador e o animal, sendo efetiva em procedimentos indolores e curtos, tais como o exame físico, coleta de amostras sanguíneas e colocação de anilhas ou sexagem[18,57]. As informações sobre técnicas de sedação em araras e emas são escassas, porém em outras espécies de aves os resultados são promissores[18,57-61].

Nas aves, um fármaco cada vez mais utilizado para a sedação é o midazolam. Este tem demonstrado produzir sedação satisfatória com mínimas alterações cardiovasculares[18,58-62]. Este fármaco pertencente ao grupo dos benzodiazepínicos, produz os seus efeitos sedativos por meio da depressão do sistema límbico e os efeitos miorrelaxantes a nível central por meio da inibição dos neurônios internunciais na medula espinal. Esses fármacos potencializam os efeitos do transmissor ácido gama-aminobutírico(GABA) nos receptores GABAa pré e pós sinápticos, sendo este um dos principais inibidores do sistema nervoso central[63]. A sua ação não induz anestesia, simplesmente sedação e os seus efeitos cárdio respiratórios são

mínimos[63]. O midazolam também produz amnesia[60], tem tempo de ação curto em comparação com outros sedativos,apresenta efeito estimulante do apetite e as recuperações após a administração são rápidas e suaves[57,59].Dentre os benzodiazepínicos, o midazolam apresenta a vantagem de ser hidrossolúvel e não ser irritante na sua aplicação intramuscular[63].Este fármaco tem sido usado para o tratamento da miopatia por captura em aves com bons resultados[38]. Outra vantagem é a facilidade de reversão por meio do seu antagonista flumazenil[18,58-61]. Porém, as dosagens utilizadas em aves são muito variadas e existem poucos estudos farmacocinéticos nestas espécies[64]. Além disso, a grande variabilidade filogenética dentro das aves faz com que a extrapolação de dosagens de uma espécie para outra seja pouco confiável[65].

Uma via cada vez mais promissora para administração de fármacos é a intranasal. Esta é pouco invasiva e proporciona rápida difusão do fármaco para o sistema circulatório, graças a rica irrigação da mucosa da cavidade nasal[66]. Quando comparada com outras vias de administração, apresenta alta biodisponibilidade e fornece altas concentrações máximas, provavelmente por evitar os efeitos do metabolismo de primeiro passo[67]. Outras vantagens são a facilidade de administração e a redução do estímulo nociceptivo em comparação à via intramuscular[18]. A administração intranasal de midazolam em aves apresentou bons resultados em vários estudos’[18,57-59,61].

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CAPITULO 2 -INTRANASAL MIDAZOLAM ON RESTRAINT INDUCED STRESS IN BLUE-AND-YELLOW MACAWS

Abstract

Sedation is a good alternative for minimizing restraint stress response in wild birds. Twenty-one blue-and-yellow macaws from a rehabilitation center were used in a randomized control (sprayed intranasal saline) - treatment (sprayed intranasal midazolam 7.5 mg/kg approximately dose) trial with two months of washout period. After application, the birds were placed inside a box for 10 min and afterwards aphysical exam (heart rate, respiratory rate and cloacal temperature) and blood collectionwere performed. Anaerobically blood samples were analyzed for PO_2, PCO_2, $ctCO_2$, SvO_2, pH, base excess, Na^+, K^+, Cai^+, Cl^- and anion gap. Hematology and biochemistry (ALT, ALP, AST, CK, LDH, glucose, cholesterol, HDL, triglycerides, total protein, lactate, urea, uric acid, creatinine and phosphorus) analyses were performed within the same day. The birds in midazolam group allowed physical exam with minimal restraint and lack of vocalization was noticed, however, most of them reacted when punctured. Results showed significantly ($p<0.05$) lower values for all physical parameters within the midazolam group. Lower lactate concentrations;base excess and Cl^- were observed, while pCO_2, $ctCO_2$ and HCO_3were higher when compared with the control group. Flumazenil intranasal application rapidly reverted midazolam sedation. Midazolam produced an attenuated metabolic acidosis and provided a mitigated restraint induced stress response. It can be concluded that intranasal sprayed midazolam is a good and safe alternative for sedation in blue-and-gold macaws, also,with minimal adverse effects. Results presented here open new perspectives on restraint techniques for this species, not only in veterinary clinics but also in fieldwork.In addition, rapid reversion with flumazenil offer further advantages upon midazolam use.

Keywords: *Ara ararauna,* biochemistry parameters, flumazenil, hematology, hemogasometry, metabolic acidosis.

1 Introduction

Blue-and-yellow macaw (*Ara ararauna*, Linnaeus, 1758) distribution range goes from southern Central America and over tropical South American countries. In Brazil, it is widely distributed mainly though Amazonia, Cerrado and Pantanal biomes[1,2]. Although classified as "Least Concern"in the IUCN red list, its population is decreasing and is one of the most traded species of its genera[3], thus the reason for being listed on CITES Appendix II.

On the other hand, the species is well appreciated as a companion animal and its presence in exotic pet clinics is common[4].

Either with wild birds or those raised as pets, the stress response to manual restraint can lead to complications including acute collapse and death, especially when they present weakness or health problems[5-8]. Restraint techniques are necessary in fieldwork, rehabilitation centers and clinics for performing multiple procedures such as blood collection, radiography, identification or physical exams.

Recently, the use of sedation techniques in birds has proved a good alternative for avoiding acute stress responses, and so minimizing risks for the animals and also for the veterinarians by providing easy and safe restraints and allowing better examinations[7,9]. This is preferred rather than general anesthesia for minor and non-painful procedures[7,10]. Anesthetized birds may present respiratory arrest, closely followed by cardiac arrest, arrhythmias, hypothermia, cardiovascular and/or respiratory depression[11,12]. Even when anesthesia is preconized, administering sedatives or analgesics before anesthesia is recommended, thus allowing for the lowering of dosages of inhaled anesthetics[12].

Benzodiazepines produce their sedative effects via limbic depression and also inhibit internuncial spinal neurons leading to muscular relaxation. These drugs enhance gamma-aminobutyric acid (GABA) transmission on GABAa pre and postsynaptic receptors[13], which represent mainly inhibitors of the central nervous system. Studies indicate that the GABAa-benzodiazepine complex has a role in the control of avian anxiety[14]. The benzodiazepines don't produce anesthesia, only sedation with minimal cardio respiratory effects. Midazolam is a benzodiazepine drug with the advantage of being water-soluble and not irritating upon intramuscular application[15]. Midazolam produces amnesia[16], rapid onset of action compared with other sedatives, no adverse effects and smooth and rapid recovery, it also presents an appetite-stimulant effect[9,17].Intranasal midazolam has been tested in various bird species demonstrating a rapid onset of action and minimal adverse effects[7,9,17,18].

Little is known about dosages and effects of intranasal midazolam in blue-and-gold macaws so the purpose of this study was to determine the effects of intranasal midazolam on restraint induced stress inthese birds, assessed by physical, hematological and biochemical parameters.

2 Materials and Methods

2.1 Birds and enclosure

The birds included in this study were from a wildlife triage center (Centro de Triagem de Animais Silvestres -CETAS-GO) located nearby Goiânia city, Goiás state, Brazil. Twenty-one adult blue-and-yellow macaws (*Ara ararauna*), 11 females and 10 males, were used. Though coming from different origins, at the time of the experiment, all the birds were forming a group in the same stage of rehabilitation for being released into the wild, this being able to fly and apparently healthy.

The birds were housed in two groups (10 and 11 animals each, mixing males and females) in two outdoor adjacent cages (5 m long, 3 m width, 2 m high) with a partially covered roof and multiple wood perches. The diet consisted of formulated extruded pellets offered early in the morning and assorted fresh fruit in the afternoon, access to water was guaranteed all day.

All procedures had the approval of The Ethical Animal Use Committee of Universidade Federal de Goiás, Goiânia; the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), Brasilia; and the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), Brasília.

2.2 Study design

Each animal was captured twice, in two different period interval (October and December of 2014) separated by two months, out of breeding season. The captures occurred always early in the morning, before offering the food and with the animals in approximately 12h starvation period. The amount of animals captured per day was a maximum offive and the same two persons always performed the procedure.

After one person captured a bird with a hand net and secured it using handling gloves, the other person sprayed 7 to 8 mg/kg (approximately 1.4to1.6 ml) of intranasal midazolam (Hipolabor, Belo Horizonte, Minas Gerais, Brasil) or the same volume of saline in the nares of the animal. For that, a human intranasal spray device was used. It sprayed 0.1 ml each time it was pressed, multiple applications being necessary in both nares in order to reach the amount of volume necessary. The treatments were randomly assigned (half receiving midazolam and the other half the saline treatment), and the dose calculated by approximation due to not knowing the exact weight of the bird. The dose was established after a pilot test in which it was adjusted gradually until reached a desirable state of sedation (this was, the bird

allowed lateral recumbence without struggling or need for further restraint). In the second period of captures, it was necessary to identify the bird by conferring the leg band number before the intranasal application in order to administer the opposite treatment to previous period, but the dose was still calculated by approximation. After the application, the animal was placed in a wooden box (68cm long, 33cm wide and 23cm tall), moved into an adjacent room and left with no light or sound stimuli for 10-15 minutes. Chasing time, administration moment, time until entering the box and ambient temperature were recorded.

The animal was removed from the box and properly handled using a towel. The bird was positioned ventrodorsally on a flat surface while properly restrained. A towel was positioned on the head for preventing visual stimuli. In most occasions, those birds receiving midazolam didn't need to be restrained during the physical exam, which was always performed by the same examiner. Heart rate (stethoscope), respiratory rate and cloacal temperature (digital thermometer, Thermoval basic, Hartmann®) were assessed. State of the feathers was classified in three levels (good, partially broken, highly broken). The body condition was observed based on the pectoral muscle mass and the amount of subcutaneous fat and classified in three levels (slim, normal, fat). Upper tract respiratory secretions, fractures, contusions or presence of external parasites were recorded.

In sequence, 0.6 ml of blood was extracted by venipuncture with a 23 gauge x 25 mm needle and a lyophilized-lithium-heparinized syringe for blood gas analysis (BD Preset®, Becton, Dickinson and Company, Plymouth, UK); this was immediately closed for avoiding air contact, placed in ice and sent to analysis. Another venipuncture was performed with a 23 gauge x 178mm scalp needle to obtain at least 3 ml of blood, this was divided in different tubes: 0.5 ml K_2EDTA 1 mg (BD Microtainer®), 0.6 ml fluoride NaFl/Na_2EDTA (BD Microtainer®) and multiple 0.4 ml lithium heparin (BD Microtainer®). Blood smears were made before any contact with anticoagulant, and fixed with methanol after drying. The locations for venipuncture were the jugular, the ulnar or the metatarsal veins, sometimes with more than one try being necessary. The time of collection of each sample was recorded. During the procedure the volume of blood extracted never exceeded the maximum recommended (10% of total blood volume, equivalent to 1% of body weight)[19].

After the venipuncture, a dose of 0.05 mg/kg intranasal flumazenil (Cristália®, São Paulo, São Paulo, Brasil) was administered in those individuals who previously received midazolam treatment. All animals were identified by placing an open inox leg band and then weighed using a digital hanging balance (Baxtran, Giropès®, Spain) and a sack. After conferring the total recovery of the bird, the animal was returned to the cage.

Blood EDTA tubes were placed in a thermic box containing ice immediately after sampling. Heparin and fluoride tubes were centrifugated (1500g) within 14 min average from blood collection, the plasma was separated from the cell clot in 0.5 ml Eppendorf® tubes aliquots and placed in ice.

2.3 Laboratorial analysis

All analyses were performed in the Clinical Pathology Laboratory of Veterinary Hospital (Universidade Federal de Goiás, Goiânia, Brasil). Those samples that presented any alteration (hemolysis or lypemia) suitable to interfere in the biochemistry results were excluded.

Blood gas analyses were performed in automated equipment (COBAS 1.2.1. Roche®) and the samples were analyzed for O_2 pressure (PO_2), CO_2 pressure (PCO_2), pH, sodium (Na^+), potassium (K^+), ionic calcium (Cai^+) and chloride (Cl^-) concentrations. Bicarbonate concentration (HCO3-), base excess (BE), oxygen venous saturation (SvO_2), anion gap (AG) and total CO_2 concentration ($ctCO_2$) were also calculated by the equipment. Calculi were adjusted for the cloacal temperature of each individual. The average time between blood collection and the analysis was 25 min and in case of exceeding 30 min, the results for blood gases were disregarded.

Plasma chemistry parameters were determined in a refrigerated automated analyzer (CM 250, Wiener Lab®, Rosario, Argentina) with commercial reagents (Labtest® - Labtest Diagnóstica S.A., Lagoa Santa, Minas Gerais, Brasil) and the recommended manufacturer quality controls were used. Plasma heparin samples were analyzed for activities ofthe enzymes alanine transaminase (ALT) (kinetic-UV method), aspartate transaminase (AST) (kinetic-U method), creatine kinase (CK) (kinetic method), alkaline phosphatase (Bowers and Mc Comb modified method), lactate dehydrogenase (LDH) (pyruvate-lactate method); concentration of uric acid (enzymatic-Trinder method), cholesterol (enzymatic-Trinder method), triglycerides (enzymatic-Trinder method),creatinine (two point kinetic method), phosphorus (Daly and Ertingshausen modified method), high density lipoprotein cholesterol (HDL) (accelerator selective detergent method), total proteins (biuret method) and urea (enzymatic UV method). Lactate (enzymatic-Trinder method) and glucose (GOD-Trinder enzymatic method)were determined in plasma fluoride in order to avoid glycolysis and stabilize blood lactate. All samples were kept in ice and analyzed in an average of 2.5 hours from collection.

Hematological parameters were analyzed using refrigerated blood in EDTA tubes. All analyses were performed in an average time of eight hours after collection. Total blood cell counts were performed manually in a Neubauer hemocytometer chamber by diluting blood 1:200 with Natt-Herrick solution[19,20]. For differential white blood cell counts, blood smears were stained with Wright's stain[21] and examined with a light microscope with x100 oil immersion lens. There were three slides per animal, so the differential counting was the mean of the three slides.

The packed cell volume (PCV) was determinate for a standard microhematocrit method. Hemoglobin was determined using hemoglobin cyanide method (Labtest®) in a semiautomatic analyzer (BIO-2000, Bioplus, Barueri, São Paulo, Brasil), always centrifuging the mixture for 5 min at 590g before the lecture. This is necessary when working with nucleated avian cells, allowing the nucleus to precipitate at the bottom of the tube to not alter the hemoglobin results[21,22].Red blood cell indices (mean corpuscular volume -MCV-, mean corpuscular hemoglobin -MCH- and mean corpuscular hemoglobin concentration -MCHC-) were calculated.

A drop of blood in a vegetal filter was sent to an external laboratory (BioTech, Goiânia, Goiás, Brasil) for sexing by polymerase change reaction (PCR).

2.4 Statistical analysis

Paired t tests for groups (control and midazolam) and periods (first and second) were performed and the differences were tested for normality with the Shapiro-Wilk test. Those values that didn't present a normal distribution range were compared using theWilcoxon paired test. Significant differences were considered with p-value <0.05.Those samples that presented any alteration (hemolysis or lypemia) suitable to interfere in the biochemistry results were excluded. Descriptive statistics were performed for observed values. Ranges were calculated using means and two standard deviations with those values that presented a normality distribution forthe Shapiro-Wilk test; and between 2.28%-97.73% quantiles, with values with no normal distribution. For values that presented differences between groups, only results from the control group were used in descriptive statistics; for those variables that didn't present differences, the mean of the two samples for each bird was used. An analysis of variance (ANOVA) for comparisons between sexes was also performed, and differences were considered with a p value <0.05.Statistical analysis was performed using the free R Core Team (2015) software[23].

3 Results

Time of procedures was similar for both groups, showing no significant statistical differences (p>0.05) between groups (Table 1).

Birds weight between treatments didn't differ either (1.02 kg for saline and 0.98 kg for midazolam, p=0.356) nor between periods (1.00 kg in first period and 1.00 in second period, p=1)only differing between sexes (p value=0.018) and it was 0.93±0.15 kg for females and 1.08±0.11 kg for males. Estimation of weight was close to the real weight, not differing significantly.

TABLE 1- Time in minutes of procedures for control group (SG) and midazolam group (MG), difference (Dif.) between groups, provability values and descriptive statistics for observed values (mean ± standard deviation - SD, lower and upper limits), n=21

Times (min) of procedures	Evaluation between groups						Observed values		
	Means		Dif.	p1	p2	p3	Mean ± SD	Lower limit	Upper limit
	SG	MG							
Chasing time	0.67	0.81	-0.14	0.452	0.010	0.464	0.74 ± 0.41	0	2
Time for treatment application	22.45	22.14	0.31	0.705	0.260	-	0.86 ± 0.64	0	3
Time inside the box	11.52	11.90	-0.38	0.672	0.468	-	11.7 ± 1.55	8.5	14.5
Duration of physical exam (since leaving box until blood sampling)	7.81	7.90	-0.10	0.897	0.053	-	7.86 ± 2.00	5.5	11.5
Time for blood gas analyses sample collection	0.33	1.00	-0.67	0.282	<0.001	0.344	0.70± 1.25	0	4
Time for second blood sample collection	8.48	7.38	1.10	0.429	0.101	-	7.91 ± 3.57	3.5	16
Total time of procedures	34.29	34.81	-0.52	0.764	0.536	-	34.55 ± 3.65	29.5	44

p1 - p-value for paired t-test; p2 - p-value for Shapiro-Wilk normality test; p3 - p-value for Wilcoxon paired test

Because estimated weight was similar to real weight, dosages were closer to those desired (7.5 mg/kg), although there was a significantly difference between sexes, being females whom received higher dosages (Table 2).

TABLE 2 - Real midazolam and flumazenil dosages received according to sex, mean ± standard deviation (SD), lower and upper limits and probability value, females n=11, males n=10

Drugs		Mean±SD	Lower limit	Upper limit	p value
Midazolam dose (mg/kg)	Females	8.5 ± 1.0	7.3	11.1	<0.001
	Males	6.5 ± 1.3	4.6	8.3	
Flumazenil dose (mg/kg)	Females	0.060	0.04	0.10	0.029
	Males	0.047	0.04	0.05	

p-value for ANOVA

When receiving midazolam, most of the birds didn't need to be restrained, they laid peacefully during the physical exam, vocalization was also absent. Even so, they needed to be restrained during blood collection because of reacting when punctured.Flumazenil produced a rapid recovery from midazolam in all animals after application. With saline treatment the birds remained alert and presented loud vocalization.

Ambient temperature during procedures ranged between 20ºC and 25ºC. Mean ambient temperature for control group was 22.45ºC and for midazolam group was 22.14ºC, not differing significantly (p=0.705).

The physical parameters (RR, HR and cloacal temperautre) are represented in table 3. Values for all physical parameters were significantly (p<0.001) lower with midazolam.

TABLE 3-Means and difference between means (Dif.) for respiratory rate, heart rate and cloacal temperature from blue-and-yellow macaws in control group (SG) and treated midazolam group (MG), provability values and descriptive statistics for observed values (mean, standard deviation - SD, range and upper and lower limits) in control group, n=21

Physiological parameters	**Evaluation between treatments**					**Observed values(SG)**				
	Means		**Dif.**	**p1**	**p2**	**Mean**	**SD**	**Range**	**Lower limit**	**Upper limit**
	SG	**MG**								
Respiratory rate	92	44	48	<0.001	0.172	92	31.1	53.8-168.8	52	196
Heart rate	443	295	148	<0.001	0.830	442.8	104.9	232.9-652.6	232	600
Temperature (ºC)	41.3	40.3	1.0	<0.001	0.605	41.3	0.52	40.27-42.34	40.4	42.0

p1 - p-value for paired t-test; p2 - p-value for Shapiro-Wilk normality test

Results for blood gas analysis are represented in table 4. In the second period, mechanical problems with the hemogasometer prevent the measurement of ionic calcium, making it impossible to perform a t paired test. PCO_2, EB, HCO_3, $ctCO_2$ and Cl^-values were lower in the control group.

TABLE 4 - Blood gas results for blue-and-yellow macaws incontrol group (SG) and treated midazolam group (MG), difference between groups (Dif.), probability valuesand descriptive statistics for observed values (mean, standard deviation - SD, range, upper and lower limits)

Blood gas parameters	**Evaluation between treatments** (n indicated in each parameter)					**Observed values** (n=21)				
	Mean		**Dif.**	**p1**	**p2**	**Mean**	**SD**	**Range**	**Lower limit**	**Upper limit**
	SG	**MG**								
PO_2 mmHg (20) F (11)	65.80	59.32	7.48	0.053	0.964	65.20	11.62	41.97-88.43	45.90	78.40
M (10)						66.45	11.07	44.31-88.59	39.90	81.30
***PCO_2** mmHg (20)	22.66	30.32	-7.66	<0.001	0.131	22.66	3.40	15.86-29.45	17.00	28.20
***$ctCO_2$**(20)	16.57	21.26	-4.69	<0.001	0.347	18.92	2.20	14.36-22.23	12.15	22.30
SvO_2 % (20) F (11)	92.13	89.68	2.46	0.220	0.168	89.51	4.62	82.41- 93.54	82.40	93.55
M (10)						92.45	3.37	85.87 - 95.34	85.15	95.35
pH(20)	7.46	7.44	0.02	0.206	0.931	7.45	0.05	7.36-7.54	7.37	7.54
***EB** mEq/L (20)	-6.31	-2.89	-3.42	<0.001	0.248	-6.31	3.00	-13.24 - 3.25	-14.20	-3.20
Na mmol/L (20)	143.71	141.72	0.02	1.000	0.898	145.05	3.49	138.07-152.03	138.60	153.20
K mmol/L (20)	3.70	3.63	0.07	0.545	0.816	3.67	0.41	2.85-4.48	3.13	4.82
iCa mmol/L	-	-	-	-	-	1.22	0.10	1.02-1.42	1.00	1.41
***Cl** mmol/L (20)	113.04	110.88	2.05	0.044	0.113	113.33	5.99	108.33 - 127.96	107.60	137.45
***HCO_3** mmol/L (20)	15.86	20.33	-4.47	<0.001	0.325	18.10	2.12	13.69-21.33	11.55	21.35
AG mEq/L (20)	14.67	13.95	2.54	0.261	0.640	13.25	12.65	-17.49 - 26.02	-35.60	30.10

p1 - p-value for paired t-test; p2 - p-value for Shapiro-Wilk normality test; p3 - p-value for Wilcoxon paired test
For those parameters that presented statistical ($p<0.05$) difference between treatments*, only the values from control group were usedin descriptive statistics
For those parameters that presented differences ($p<0.05$) between sexes observed values are presented separately by sex (females -F and males -M)

Results for biochemical parameters are presented in Table 5. Only lactate values were significantly ($p<0.05$) lower in the midazolam group.

TABLE 5 - Biochemistry valuesfor blue-and-yellow macaws incontrol group (SG) and treated midazolam group (MG), difference between groups (Dif.), probability valuesand descriptive statistics for observed values (mean, standard deviation - SD, range, upper and lower limits)

Biochemistry parameters	**Evaluation between treatments** (n indicated in each parameter)						**Observed values** (n=21)				
	Means		**Dif.**	**p 1**	**p2**	**p3**	**Mean**	**SD**	**Range**	**Lower limit**	**Upper limit**
	SG	**MG**									
ALP UI/L (18)	265.79	246.02	2.69	0.965	0.003	0.417	304.82	303.72	92.06 - 1092.31	87.75	1308.9
ALT UI/L (21)	50.79	44.62	6.18	0.708	0.067	0.708	47.71	14.76	18.18-77.23	25.97	85.35
AST UI/L (21)	146.15	165.26	-19.12	0.153	0.007	0.355	155.70	36.50	82.71-228.70	93.02	239.80
Cholesterol mmol/L (20)	3.23	3.34	-0.17	0.428	0.473	-	3.25	0.72	1.81-4.69	1.88	4.99
CK UI/L (20)	361.56	307.35	41.95	0.291	0.096	-	343.14	229.04	51.61-858.29	15.40	1027
Creatinine µmol/L (17)	6.19	3.54	1.77	0.667	0.010	0.673	4.42	7.96	0.00 - 24.75	0.00	31.82
Glucose mmol/L (20)	15.99	15.64	0.6	0.440	0.136	-	15.85	1.81	12.23-19.48	11.98	18.74
HDL mmol/L (20)	1.89	1.93	-0.07	0.681	0.068	0.498	1.89	0.35	1.19-2.59	1.3	2.47
***Lactate** mmol/L (20)	10.9	6.68	4.47	0.002	0.397	-	10.9	4.85	5.27 - 22.14	4.66	25.97
LDH UI/L (20)	278.70	265.59	10.47	0.774	0.269	-	282.65	104.34	73.97-491.33	130.15	469.55
Phosphorus mmol/L (21)	1.21	1.29	-0.06	0.578	0.314	-	1.26	0.28	0.69-1.83	0.86	1.82
Total plasma protein g/L (20)	43.2	40.3	2.2	0.336	0.411	-	41.6	6.4	28.8-54.3	30.4	54.5
Triglicerydes g/L (21)	0.95	0.92	0.03	0.824	0.088	0.476	0.94	0.37	0.19-1.68	0.26	1.79
Urea mmol/L (19)	0.22	0.28	-0.06	0.434	0.089	0.5306	0.25	0.28	0.00 - 0.85	0.00	1.00
Uric acid mmol/L (21)	0.33	0.22	0.11	0.085	0.250	-	0.27	0.13	0.01- 0.54	0.04	0.55

p1 - p-value for paired t-test; p2 - p-value for Shapiro normality test; p3 - p-value for Wilcoxon paired test
For those parameters that presented statistical ($p<0.05$) difference between treatments*, only values from saline treatment were used in descriptive statistics

Results for hematologic parameters are presented in table 6 andthere weren't statistical significant differences between treatments (p>0.05).

TABLE 6 - Hematology parameters for blue-and-yellow macaws in control group (SG) and treated midazolam group (MG), difference between groups (Dif.), probability values; and descriptive statistics for observed values (mean, standard deviation - SD, range and upper and lower limits) in both groups, n=21

Hematology parameters		**Evaluation between treatments**						**Observed values**				
		Means		**Dif.**	**p 1**	**p2**	**p3**	**Mean**	**SD**	**Range**	**Lower limit**	**Upper limit**
		SG	**MG**									
RBC $10^6/mm^3$		2.44	2.63	-0.19	0.1831	0.431	-	2.54	0.36	1.83-3.24	1.86	3.45
PCV %		41.00	40.52	0.40	0.595	0.527	-	40.7	3.1	37.7-46.9	37.5	48.0
Hemoglobin g/dL		11.31	11.24	-0.01	0.992	0.119	-	11.24	1.27	8.7- 13.8	8.7	13.8
MCV fl		168.15	158.43	9.70	0.260	0.453	-	163.1	15.2	132.8-193.5	138	194
MCH pg		46.75	44.14	2.35	0.440	0.825	-	45.1	7.5	30.3-60.0	32	60
MCHC %		27.75	27.67	-0.05	0.967	0.100	-	27.7	3.0	21.7-33.7	22	35
Leucocytes /mm^3	F	10607	10964	-357	0.874	0.007	0.578	13590	6928	6154-26132	6125	27125
	M							7700	3371	958-14443	3375	15000
Heterophils /mm^3	F	7046	7571	-212	0.918	0.013	0.498	9627	5635	2951-19289	2942	19801
	M							4421	2713	965-9811	790	10663
Lymphocytes /mm^3		2899	3003	-35	0.954	0.621	-	2916	1051	813-5019	1508	4821
Monocytes /mm^3		510	579	-51	0.779	0.083	0.784	534	517	134-1891	125	2457
Eosinophils /mm^3		66	57	9	0.765	0.522	-	62	56	5-201	4	223
Basophils /mm^3		88	71	21	0.388	0.003	0.870	100	112	8-367	7	528
H/L	F	2.78	2.38	0.51	0.491	<0.001	0.596	3.36	2.06	0.98 - 7.35	0.95	7.51
	M							1.62	0.80	0.02 - 3.23	0.52	3.22
Thrombocytes $10^4/mm^3$		3.22	3.42	-0.20	0.72	0.576	-	3.32	1.77	1.35-7.26	1.35	7.65

p1 - p-value for paired t-test; p2 - p-value for Shapiro normality test; p3 - p-value for Wilcoxon paired test
For those parameters that presented differences between sexes observed values are presented separately by sex (females -F- n=11; and males -M- n=10)

When comparing periods and without considering the treatment, some variables presented significant differences, those are presented in table 7. During the second period most of the birds presented parasites (feather mites) that weren't reported during the first period.

TABLE 7 - Parameters for blue-and-yellow macaws presenting significant differences among periods with paired t-test, period means, difference between periods and probability values

Parameters	Means		Difference	p1	p2	p3
	First period	Second period				
Ambient temperature (ºC)	23.7	21.0	2.74	<0.001	0.115	-
Total time (min)	32.4	36.7	-4.3	0.006	0.535	-
Cloacal temperature (ºC)	41.3	40.3	0.91	0.003	0.161	-
Leucocytes (/mm^3)	7833	13738	-5904	0.003	0.009	0.003
Heterophils(/mm^3)	5314	9391	-3850	0.048	0.006	0.006
Lymphocytes (/mm^3)	2015	3932	-1891	<0.001	0.065	-
Monocytes (/mm^3)	316	784	-459	0.005	0.003	0.001
Eosinophils (/mm^3)	108	14	96	<0.001	0.059	<0.001
Total plasma protein (g/L)	41	47.5	-7	0.022	0.160	-
LDH (UI/L)	236.2	306.1	-74.5	0.03	0.096	-
AST (UI/L)	141.1	173.2	-35.1	0.015	0.003	0.005
ALT (UI/L)	14.2	78.9	-64.9	<0.001	0.800	-
Glucose(mmol/L)	14.88	16.75	-1.75	0.016	0.032	0.015
HDL (mmol/L)	2.22	1.61	0.59	<0.001	0.191	-
Phosphorus (mmol/L)	1.13	1.36	-0.23	0.023	0.044	0.040
PO_2 (mmHg)	56.3	68.7	-12.4	<0.001	0.917	-
SVO_2%	88.2	93.6	-5.5	0.003	0.06	-
Na (mmol/L)	147.9	142.2	5.6	0.015	0.004	0.022

p1 - p-value for paired t-test; p2 - p-value for Shapiro normality test; p3 - p-value for Wilcoxon paired test

4 Discussion

The birds included in this study constituted a heterogeneous population sample, mainly because of multiple origins. Some of them were raised illegally in captivity, others suffered illegal trade and some came injured from the wild, all ending in CETAS-GO rehabilitation center for medical care and release training. This group specifically was housed

in big cages for flight improvement, but some individuals until were unable to perform ideal flights and had different grades of feather damage. All these circumstances may produce chronic stress at some level[24-26]. Nevertheless, when bird restraint is needed in field conditions or when dealing with ill patients, the animals might be far from being in good health and welfare. From this point of view, the individual variability in this study was useful in determining if midazolam application could be of beneficial use in fieldwork. The decision of performing a comparison using a paired t test was based on minimizing this individual variation.

For the same reason, reference range values presented here must be interpreted carefully when used for comparisons with healthy or pet birds. However,it might be useful information for rehabilitation centers with animals in similar conditions.

In the present experiment the midazolam dosage was established after a pilot study using blue-and-yellow macaws from the same rehabilitation center, but not the same individuals used in the study. The starting dosage was 1 mg/kg and it was gradually increased until reaching 7.5 mg/kg, when the bird permitted dorsal recumbency. Midazolam dosages in birds are wide and some authors routinely use the same dosages (2 mg/kg, IN) for all pet birds[7]. This intranasal dosage was successful in amazon parrots, providing sedation within 3 min that lasted for 25 min[18]. Some formularies recommend dosages between 0.5 to 3 mg/kg for birds[27], however higher intranasal dosages have been used successfully and without any reportable side effects. In pigeons an IN midazolam dosage of 6.5 ± 1 mg/kg produced dorsal recumbency for 23.4 ± 3.7 min within 3 min, and the length of sedation effects was approximately 82 minutes[9]. Dosages of 2, 4 and 6 mg/kg were used in quail with 10 min of onset action and providing good sedation with both higher dosages, although action length was improved with 6 mg/kg[16]. Dosages of 7.3 mg/kg have been used in ring-necked parakeets[28] and higher dosages have been used without any adverse effects in canaries (12.5-15.6 mg/kg) and finches (13 mg/kg)[17,29].

Stress responses vary depending on the origin and character of each individual, for example wild or pet birds[14,30,31], as so, the need for higher dosages in this study might be due to these temper differences. Differences in dosages between sexes in this experiment are the result of the lack of knowledge of animals' weights before the treatment administration. This experiment design was intended to minimize stress response that could have been derived from previous weighing. Females presented lower weights that were overestimated. Even with the highest dosage (11.1 mg/kg) no side effects were reported.

In this study,IN midazolam at 5.2 to 9.1 mg/kg provided a good level of sedation in blue-and-yellow macaws. However, reduction of visual stimuli and a quiet environment were always provided. It was noticed that the noxious stimuli of blood collection reduced the sedation effect, producing some struggling movements; nevertheless the restraint was still facilitated when compared with thecontrol group. This could be explained by the lack of midazolam analgesic effects[32]. Some authors recommend a combination of midazolam and butorphanol for macaws to achieve a sufficiently deep level of sedation[7], thus also providing analgesic effects[33].

One adverse effect reported with midazolam administration is regurgitation, mainly in macaws[7]. This adverse effect was also related in pigeons with either IN and IM midazolam application[34]. In the present study this was reported only during the pilot period but it was reverted with flumazenil administration. It is known that the drug is mainly metabolized in the liver but few studies have been made focusing on midazolam pharmacokinetics[35], and further research on this area should be done.

Exact time of sedation reversal after flumazenil application wasn't reported in this study but all animals were returned to the cage in less than ten minutes after flumazenil administration and apparently completely recovered. A rapid reversal effect without re-sedation has been reported in other studies in which flumazenil was used either IN or IM with dosages that ranged between 0.01 to 0.1 mg/kg. Onset time on those studies varied from 2 to 10 minutes[7,16-18,28]. Flumazenil administration is always recommended because even though appearing slightly sedated while manually restrained, once the stimulus is discontinued, the sedative effects might increase, compromising the sensory and motor functions[7]. In humans, flumazenil didn't completely antagonize all the cognitive effects produced by midazolam[36]. Flumazenil administration appeared to reverse paradoxical reactions in humans and still retaining midazolam sedation and amnesia effects[37].

Advantages of intranasal drug delivery are the easy administration, high bioavailability, drug absorption and reduced pain when compared to intramuscular administration. It also avoids elevation of muscular enzyme activity[7], which in this case study was necessary for avoiding any alteration that could mask any stress effects. The highly irrigated epithelium of nasal cavity permits a rapid diffusion of the drug into the circulatory system. This route also ensures the avoidance of first-pass metabolism, as nasal venous drainage ends into the right heart chambers. There are several factors that influence IN drug absorption, either related to anatomical nasal structures or physical drug properties[38], but in humans, IN midazolam presented high bioavailability (82%)[39].

In macaws the intranasal administration can present some problems due to the volume of drug needed, producing sneezing during application and consequently incomplete drug delivery[7]. Due to this, and after observing this problem in the pilot study (in which the drug was delivered using a syringe) a spray mechanism was used. In humans, it has been reported that aerosolized rather than drip administration of intranasal midazolam may enhance drug delivery[40]. Using the spray delivery was useful in the present study, reducing sneezing and resulting in satisfying drug delivery. A good alternative could have been the use of a higher drug concentration presentation (e.g., midazolam 50 mg/mL; Zoopharm, Windsor, CO USA)[7], which is available in some countries but not in Brazil.In other studies that used IN midazolam in birds no clinical effects such as nasal discharge or respiratory sounds were reported[17,18]. In humans, a burning effect was described when a higher concentration formula of midazolam (50 mg/ml) was used[39]. Radiography with contrast media showed that with intranasal application the substance remained in the nasopharyngeal region in canaries, although viscosity of this media could differ from that of midazolam[17]. There wasn't reports of aspiration or ingestion of intranasal midazolam[18]. In medicine, new formulas for improving IN midazolam uptake are being investigated[41].

In the present study, birds presented lower RR, HR and cloacal temperatures with midazolam administration. Besides, lack of vocalization with midazolam use was reported, fact that was also recorded in amazon parrots[18]. Attenuated physiological stress response to restraint has been reported in other species with intranasal midazolam use, mainly characterized for slower and lower increases in respiratory and cardiac rates, also reducing the raising of cloacal temperature[18]. When exposed to stressful stimuli, the sympathetic nervous system together with catecholamine release from adrenal medulla, mediate a rapid response known as fight-or-flight. This causes a rise in the HR, cardiac output and arterial pressure, the irrigation of skeletal and cardiac muscles, lungs and brain increases and detrimentally, visceral organs, kidneys, skin, and conjunctive tissues experience vasoconstriction[42,43].

Results obtained in this study were similar to those described in amazon parrots, in which increases in body temperature within 4 min of restraint were reported (mean values were 43.9ºC ±1.0ºC). Those changes were correlated with increases in RR but no significant changes in HR were observed[44]. Herraez et al.[45]suggested that variations in HR, rather than absolute HR, could be more sensitive measures for assessing stress. Other studies reported fever and tachycardia in birds after simple handling[8,46]. Increases in body temperature after handling have been attributed to a fever-like response rather than hyperthermia[8,46] this explaining the effect of midazolam inhibiting central nervous responses and allowing lesser

fever responses[47]. Increased RR have been related to heat dissipation and also sympathetic stimuli[43].

Although observing higher cloacal temperatures in the control group, mean values were lower in our case study when compared to previous literature[8,46]. This is probably due to the limit upper range of the digital thermometer used in our study (42ºC). Also, in certain individuals with high HR, assessment was difficult and approximate rather than exact values were recorded. The use of other equipment could have provided more precise HR values[48,49]. Without these technical limitations the differences between two treatments could possibly have been more pronounced, because high HR and cloacal temperatures were found in the control group.

If only the control group is regarded, a wide range in HR values is observed. In falcons, variations in HR upon individuals depending on the frequency of handling were detected, suggesting that animals used to handling presented reduced stress responses[49]. In the present case study, the birds multiple origins could be the reason for these differences, depending on whether they are used to being handled.

In this study hemogasometry and plasma biochemistry were used for indirect evaluation of stress capture effects, since blood gas analysis permit to assess respiratory and metabolic effects of restraint[50]. Data for blood gas reference ranges in avian species are sparse and yet not deeply studied. It's well known that general anesthesia produces cardiovascular and respiratory depression that alters blood gas values[11], also, the stress response of manual restraint in non-anesthetized birds can alter blood gas values[50,51], reasons which justify the difficulty of establishing avian blood gas reference ranges. Although not conclusive, some studies correlated blood lactate levels with the difficulty of capture, this associated to high stress levels in flamingos[52]. Serum lactate concentration is a good predictor of muscular hypoxia and fatigue; main factors of the pathophysiology of capture myopathy[42]. There islittle information about normal lactate levels in avian plasma, however, compared with other studies in different avian species, present results in thecontrol group are high[50,52].

In the present experiment, treatments differed significantly not only among lactate but also pCO_2, $ctCO_2$, BE, HCO_3 and Cl values. The interpretation of those values must be done with a whole understanding of metabolic and respiratory systems involved[51,53]. With stress related restraint, anabolic metabolism increases, raising blood lactate levels. This acid compound compromises the stability of blood pH, leading to metabolic acidosis and activating compensatory mechanisms. A decrease in PCO_2 in attempt to blow off the excess of acid will occur. Bicarbonate and BE values will be low, thus indicating a metabolic origin

of acidosis, Cl ions will rise in an attempt to compensate HCO_3 decrease and AG will be higher due to lactate.

Another hypothesis for explaining blood gas differences between treatments could be a respiratory depression caused by midazolam.In birds, both inspiration and expiration are active processes that require muscle activity; as such, the depressing effect of midazolam on muscle function could negatively affect ventilation[54].This would raise PCO_2 values, leading to respiratory, rather than metabolic, acidosis.However our PCO_2 data is within ranges when compared with other published values of non-sedated birds[50,51], suggesting that midazolam didn't notably affect respiratory function. Also, regarding venous PO_2 values, no significant differences between treatments were observed, and can be considered high when contrasted with previous studies[50]. In medicine, formulas for extrapolation of arterial values from venous PCO_2, pH and HCO_3 values have proved useful but no correlation was detected for PO_2 between venous and arterial samples[55]. However, in avian medicine, no studies on this subject have been made. As such, for better knowledge of how midazolam affects respiratory function, an arterial sample collection would have been more useful[53,56].

Blood gas analyzers have a conversion formula within its software to correct for core-body temperature differences. The impossibility of detecting temperatures higher than 42ºC of the thermometer used in the present study could have influenced blood gas results. Nevertheless, this correction formula was established for human individuals and it is not known whether this formula can be accurately used in avian species.Also, most of published blood gas values for avian species have been determined using a portable point care analyzer[28,42,52], different from the one used in this study. Correlation between these portable analyzers and the standards is high, except for ionized calcium and potassium[57]. Blood gas values of the control group are similar to those reported for other avian species[28,58]. Nevertheless the role of restraint stress and lactate values haven't been discussed before in assessment of blood gas values, and here, those demonstrate to have an important influence.

Lactate levels after exercise flights have been used to assess release fitness in rehabilitated raptors[59]. Based on those values other authors concluded the safety of mist net capture in passerine and columbiform birds[50]. Mean lactate values in the present study are similar to those published previously[50,52,59], however the highest value reported in this study (25.97 mmol/L) is higher than any reported before and occurred in the control group. Reference ranges for lactate values of many avian species are not available but it's known that lactate levels elevate rapidly due to exercise and restraint[59]. Also, return within basal levels is rapid, showing decreases after 10 min post exercise[59]. How lactate could be used as a marker

of stress induced by restraint should be further studied. However, restraint techniques that produce high elevations of this compound should be reviewed.

In the present experiment, no significant differences for enzyme activities between treatments were detected. This could be due to the short time between physical restraint and blood collection (5 to 11 min), insufficient for allowing increases in blood enzymatic activities. Rises in plasma enzyme activities have been related to capture stress in some birds[60], being CK, AST and LDH the most representative ones. In mallards the elevation appeared to be related to the length of time that the birds were restrained and the capture method used[61,62]. In houbara bustards, elevations in CK and LDH were attributed to stress related capture. Elevated levels were detected 30 min after handling, reaching maximum activities after 24h and not returning to normal ranges until 14 days later[63]. Increased CK activity levels have been associated with impaired integrity of muscular membranes, associated with oxidative injury elicited by stress[64]. Elevations of AST, CK, ALT, LDH and potassium are presumed in diagnosis of extreme cases of capture stress such as capture myopathy[65]. In greater sandhill cranes with capture myopathy, peak enzyme activities occurred within 3 days after capture, and decreased only 10 to 17 days later, except AST activities that remained elevated, the authors suggested that enzyme activities could be used as indicators for risk development of capture myopathy and for survival prognosis[66,67].

In the present study midazolam treatment didn't produce differences in plasma enzyme activities within approximately 34 min of manipulation. Nonetheless, it could be expected that if midazolam had an attenuation stress effect, control group would present higher enzyme activities. Another explanation for the lack of plasma enzyme activity rises in control group could be the magnitude of stress, which could have been weak for generating such response; however, this hypothesis is less probable because of the remarkable differences in blood gas values and lactate. Variations in those metabolites might be faster than elevations in enzyme activities.

Stress response also is characterized by increased glycolysis in muscle and liver, thus resulting in elevations in glucose blood levels. Catecholamine release increase protein and lipid catabolism and causes plasmatic increases of amino acids, glycerol, fatty acids and glucose as well[68]. In the present experiment such changes weren't detected.

Broad value ranges obtained in this experiment show the heterogeneity of the population studied and even though these values cannot be regarded as reference ranges for the species, when compared with values previously published for *Ara ararauna*[21,69-78]the results of the present study are roughly within ranges. The most notable differences were high

values for CK and ALP activities, also cholesterol and triglycerides were below published ranges. However higher elevations in CK were reported in will tundra swans and attributed to stress capture[79] and it is well known that pronounced raises of these enzyme activities due to stress during blood collection are common[80]. ALP function is related to energy intake for ion exchange between cell membranes and differently from other enzymes, its elevations are related to rises in synthesis rather than cell damage[81]. In this case, elevations could be due to bound healing therefore some animals could have suffered from trauma. Alimentary restriction in chickens reduced lipid, cholesterol and HDL concentrations[82]. Low values of those metabolites in some of individuals could be explained for the rehabilitation process ongoing thru and maybe dominance behaviors within the group. Moreover, chronic stress due to conditions of a rehabilitation process might cause alterations in biochemical parameters[83]. In humans, fasting time is known to produce variations in the lipid testing[84]. In birds the influence of this factor has not been tested and differences observed here could reflect variations in fasting time between studies.

Is important to consider when comparing values that most biochemistry tests realized in this experiment were performed in heparinized plasma (except for glucose and lactate) rather than serum. The need for a sufficient sample size was the reason for this decision. Manufacturers' instructions allow both serum and plasma, for most of the tests, however, for total protein, triglycerides and LDH, serum, rather than heparinized plasma is indicated. Plasma total protein includes fibrinogen; as such, high values are expected when compared with serum[85]. For triglycerides, heparin enhances the activation of lipoprotein lipase, producing decreases in their values[86], however the short time between sampling and analysis might have mitigated this alteration. Falsely increased LDH concentrations can occur by contamination with erythrocytes and platelets, which contain high concentrations of this enzyme[87].

In the present experiment, no significant differences for hematological parameters between treatments were detected.Stress response causes leukocyte mobilization from the marginal pool to the circulation, causing a leukocytosis with heterophilia, lymphopenia and eosinopenia in birds[88]. Release of glucocorticoids causes an influx of heterophils from bone marrow and minimizes their way out to other compartments, contrary, lymphocytes adhere the endothelial wall and migrate to tissues. This response varies within species and due to that, increases in heterophil/lymphocyte ratio is considered a better stress indicator. These changes are detectable from 30 min to 3 hours post-stressful event, decreasing 24 h after

stimuli removal[89,90]. The time delay between the stressful event and the hematological changes could be the reason for the lack of differences between treatments, in this case.

Total leukocyte counts, specially heterophils, but also basophil, monocyte and lymphocytes are high if compared with published literature [21,69,70,72]. It has been suggested that alterations in the leucogram might be better indicators of long-term stress than corticosterone dosages (which returns to basal levels rapidly) because it's rapid, long lasting and highly dynamic[89]. High heterophil numbers are related to infection, inflammation, stress, toxicities, trauma and leukemia, also infections caused by mycobacteria, chlamydia and aspergillum. Blood smears didn't present toxic changes in heterophils, which generally is related to bad prognosis[91]. Nevertheless, a small amount of reactive blast lymphocytes was observed. This is common in cases of immunologic stimulation[19,91]. A reduction in eosinophils is expected in stress situations[89], however basophilia could be present in trauma or hyper immune reactions, as in birds they release histamine[92]. Monocytosis is common in chronic inflammations[91].

Interestingly, if comparing between periods a rise in leucocyte counts is noted, in addition to raises in some enzymatic activities and biochemistry values. Those differences couldn't have been related to stress accumulated response from the first collection period, because the washout period was sufficiently long.

Also, during the second period, the infestation with feather mites was noticed.Mite infestations have been documented in captive psittacine birds in brazil, with the presence of multiple mite species[93]. Ectoparasites are common in birds and in low quantities they don't cause a major problem. However, in large numbers they can compromise bird's health and, in the case of blood-sucking ectoparasites, lead to anemia[94] and even death[95]. In the present study, the identificationof feather mites wasn't accomplished.

Decreases in ambient temperatures were also observed in the second period. Even though this temperature difference wasn't pronounced, it can be related to transition between dry and rainy seasons in Cerrado biome. In this region, contrary to temperature, precipitation presents a marked seasonality and it concentrates between October and March[96]. The fact that the first period of the experiment took place during the beginning of this seasonal transition could somehow affect the birds and reflected in period differences.

A longer captivity time in the second period could also play a role in parameters' variations.All those events could contribute to compromising the animals’ health, altering hematologic and biochemistry parameters, masking possible treatment effects.

The present study demonstrates the efficacy of intranasal sprayed midazolam in mitigating restraint-induced response in blue-and-gold macaws. This drug attenuated metabolic acidosis, physical parameters related to stress and allowed an easy and safe restraint, with minimal adverse effects. It can be concluded that intranasal sprayed midazolam is a good and safe alternative for sedation in this specie. Results presented here open new perspectives on restraint techniques for this species, not only in veterinary clinics but also in fieldwork, furthermore, rapid reversion with flumazenil offer further advantages upon midazolam use.

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CAPITULO 3: MIDAZOLAM ON RESTRAINT INDUCED STRESS IN GREATER RHEAS

Abstract

Stress response elicited by restraint can be life threatening, being capture myophaty one of the most serious complications. A good alternative for minimizing stress handling response in birds is sedation. In this study fourteen greater rheas were used in a randomized control (saline) - treatment (1 mg/kg IM midazolam) trial with a one and a half month washout period. After application, a standardized 10 min waiting time was established and the physical parameters (HR, RR, T^a) and blood collection for hematological (RBC, thrombocytes, leucocytes, heterophil, lymphocyte, basophil, eosinophil and monocyte counts; H/L ratio; PCV; hemoglobin concentration and RBC index) and biochemistry (LDH, ALT, AST, CK, ALP plasma enzyme activities; triglycerides, cholesterol, HDL, glucose, total protein, urea, uric acid, creatinine, phosphorus, lactate, Na^+, K^+, Cai^+ and Cl^- plasma concentrations) analyses were performed. The only parameter that presented significant differences ($p<0.05$) between groups was RR, being higher with midazolam application. Midazolam produced an inconstant response that varied among individuals, some of them showing calmbehavior and others presenting ataxia and struggling. Insufficient dosage or inconstant IM drug absorption could be the reason for the observed results. IM midazolam at 1 mg/kg dose didn't provide acceptable sedation in captive greater rheas. Hematological and biochemical parameters presented here might be used as reference values for captivity adult greater rheas in central Brazil, out of breeding season.

Keywords: capture myopathy, clinical biochemistry, hematology parameters,reference ranges, *Rhea americana*.

1 Introduction

The greater rhea (*Rhea americana*, Linnaeus, 1758) is classified within the order Struthioniformes, specifically in the family Rehidae. These birds are often grouped with other flightless birds such as ostriches, emus, cassowaries and kiwis beyond the term "ratites". Contrary to once believed, this species are now considered to have originated from unrelated groups that have adapted to a specialized terrestrial lifestyle[1]. The distribution of greater rheas ranges from northeast and southeast Brazil, east Bolivia, Paraguay, Uruguay and northeast and east Argentina. Despite this wide distribution range, their populations are decreasing, mainly due to habitat reduction and fragmentation consequence of agriculture and cattle-

ranching, reasons for being classified as "near threatened" in the red list of threatened species[2].

In the last few years, this species has been reared in captivity for conservational and also commercial purposes[3]. Regardless of its ultimate objective, captivity rheas need to be handled for medical or management purposes. Restraint procedures in wild animals are always risky for both the handler and the animal. In fieldwork, longer handling times have been related to higher mortality rates during the first week after capture[4]. Stress response elicited by restraint can be life threatening for birds, capture myopathy (CM) being a common cause of demise[5-7]. CM also known as exertional myophaty or exertional rhabdomyolysis is a multifactorial syndrome originatingfrom acute stress response[8].

Certain species seem to be prone to suffer from CM and birds with large muscular mass and long-legged, such as ratites, are more susceptible[8]. It is believed that the activation of sympathetic nervous system and the hypothalamic-pituitary-adrenal axis triggers a metabolic cascade that leads to tissue hypoperfusion and hypoxia ending with collapse and death[9]. Different authors have classified CM depending on signals, lesions and time of presentation, but generally, ataxia, dyspnea; different levels of myopathy (leading to paralysis and myoglobinuria) and cardiac failure are present[9]. Those signals can appear immediately, hours or days after capture[8]. In all cases high muscular enzyme activities are present. Confirmatory histopathology lesions are muscular acute or sub acute degeneration (either in skeletal and cardiac muscles)[10], usually, tubular renal damage commonly associated with myoglobinuria[11] and necrosis in renal tubes, brain, liver, pancreas, heart, spleen, lymph nodes and adrenal glands can occur[8].Prognostic of CM is bad and treatment is complicated and long lasting[12,13]. For this reason a great effort has been focused on prevention; and the use of tranquilizers and neuroleptic drugs has proved useful in reducing stressful responses[8,14].

General anesthesia is a useful restraint method when dealing with ratitesbut complications such bradycardia, hypothermia and apnea, can occur[15]. Recently, the use of sedation techniques in birds has been proved as a good alternative for avoiding acute stress responses and also avoiding risks related to general anesthesia[16,17]. Midazolam is a benzodiazepine drug that produces sedative effects via enhancing gamma-aminobutyric acid (GABA) transmission, producing limbic depression and muscular relaxation[18]. It has been stated that GABAa-benzodiazepine complex plays a role in the control of avian anxiety[19]. The main advantages are water-solubility that produces no irritation on intramuscular application, minimal cardio respiratory effects[20], amnesia[21], appetite-stimulant effect, rapid onset of action

and smooth and rapid recoveries[22,23]. In Bar-tailed godwits, midazolam was used as part of CM treatment with good results[14].

Stress response, even when it doesn't end up with CM, produce physiological, hematological and biochemical changes[8,24-31]. Present study tries to evaluate the effect of midazolam on restraint stress response in greater rheas, by evaluating differences in physiologic, hematologic and biochemical parameters.

2 Materials and methods

All procedures were approved by The Ethical Animal Use Committee of Universidade Federal de Goiás, Goiânia, the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), Brasília, and the Agência Muincipal do Meio Ambiente of Goiânia (AMMA-GO).

Fourteen captive adult rheas (*Rhea americana*), 7 females and 7 males, from Goiânia Zoological Park (Goiânia, Goiás, Brasil) were randomly assigned in two groups, either receiving midazolam (Hipolabor, Belo Horizonte, Minas Gerais, Brasil, 1 mg/kg, IM) or equivalent volume of saline solution (control group). After a washout period of one and a half month, each animal was recaptured and the treatments inverted. All the experimentswere done out of breeding season. The day before the beginning of the experiment, all animals were weighed on anelectronic balance. All captures were performed during the morning, between 8 to 11 am, with an average of 4 animals per day,with the birds in a 12h fasting period.

The procedure consisted firstly in isolating a bird from the group in a closed room; the reading of its microchip was made without any restraint. Afterwards, the animal was encircled between the room wall and a handmade restraint artifact (figure 1) that partially restrained the bird movements but with two windows that allowed access to the animal. Immediately, either the midazolam or saline was injected in the biceps brachii muscle, avoiding the renal portal system. After a 10 min waiting time (established for the drug to act), and still with the animal restrained, heart rate (HR), respiratory rate (RR) and cloacal temperature (T^{a}) were assessed. Some birds didn't tolerate the partial restraint, constantly moving and kicking. Those animals (a total of 7) were allowed to freely move around the room but with a hood blocking its vision, in those birds the physical parameters were measured without any further restraint. In order to minimize different responses due to

variations in the restraint method instead of due totreatments, the same restraint technique was repeated in the second experiment period. After assessing physical parameters, the restraint artifact was removed and the bird was hooded. Immediately the bird was completely restrained on the floor for the blood collection, always ensuringthe bird stayed in a sitting position, with legs folded, and controlling the amount of pressure to allow the bird to breathe properly. Blood was collected by jugular venipuncture with a 21gauge x 25 mm needle and was divided in a 4 ml lithium heparin tube (BD Vacutainer®, Becton, Dickinson and Company, Plymouth, UK), one 0.6 ml fluoride NaFl/Na2EDTA tube (BD Microtainer®) for biochemistries and one 0.4 ml lithium heparin (BD Microtainer®) for hematology. Blood smears were made before any contact with anticoagulant, and fixed with methanol after drying. In some cases, more than one puncture was needed in order to reach the amount of blood, each time the syringe and the needle were changed. The blood was immediately placed within the tubes to avoid coagulation. Immediately after blood collection the animal was released. Chasing time (until entering the contention cage), moment of treatment application, time spent on physical exam, time with the hood on, length of total restraint, time of blood collection and number of punctures were recorded.

FIGURE 1 - Restraint artifact used in greater rheas

The tube with 0.4 heparinized blood was refrigerated until analized. The 4 ml heparinized sample and the fluoride sample were centrifuged 1000g for 10 min within an

average of 13 min since the moment of collection. The plasma was separated in 0.5 ml Eppendorf® tubes and stored in ice.

Samples were processed within the same day of collection in the Multiuser Laboratory of Pós Graduation Program (Universidade Federal de Goiás, Goiânia, Brazil).

Plasma chemistry parameters were determined in a non-refrigerated automated analyzer (CM 250, Wiener Lab®, Rosario, Argentina) with commercial reagents (Labtest® - Labtest Diagnóstica S. A., Lagoa Santa, MG) and the recommended manufacturer quality controls were used. Plasma heparin samples were analyzed for activities of alanine transaminase (ALT) (kinetic-UV method), aspartate transaminase (AST) (kinetic-UV method), creatine kinase (CK) (kinetic method), alkaline phosphatase (Bowers and Mc Comb modified method) and lactate dehydrogenase (LDH) (pyruvate-lactate method); concentrations ofuric acid (enzymatic-Trinder method), cholesterol (enzymatic-Trinder method), triglycerides (enzymatic-Trinder method), creatinine (two point kinetic method), phosphorus (Daly and Ertingshausen modified method), high density lipoprotein cholesterol (HDL) (accelerator selective detergent method), total proteins (biuret method) and urea (enzymatic UV method). Lactic acid (enzymatic-Trinder method) and glucose (GOD-Trinder enzymatic method)were determined in plasma fluoride in order to avoid glycolysis and stabilize blood lactate. All samples were analyzed in an average of 7 hours from collection except for triglycerides, cholesterol, total protein, uric acid and phosphorus that were determined after 3 to 4 months of storage at -20ºC.

Electrolyte determination of sodium (Na^+), potassium (K^+), ionic calcium (Cai^+) and chloride (Cl^-) was performed with automated equipment (COBAS 1.2.1. Roche®) in plasmaafter 3 to 4 months of storage at -20ºC.

Hematological parameters were analyzed using blood in 0.4 ml lithium heparin tubes. All analyses were performed in an average time of 8 hours after collection. Total blood cell counts were performed manually in a Neubauer hemocytometer chamber by diluting blood 1:200 with Natt-Herrick solution[32,33]. For differential white blood cell counts, blood smears were stained with Wright's stain[34]and examined with a light microscope with x100 oil immersion lens. There were three slides per animal, so the differential count was the mean of the three lectures.

The packed cell volume (PCV) was determinate by a standard microhematocrit method. Hemoglobin was determined using hemoglobin cyanide method (Labtest®) in a semi automatic analyzer (BIO-2000, Bioplus, Barueri, São Paulo, Brasil), always centrifuging the mixture for 5 min at 590g before the lecture. This is necessary when working with nucleated

avian cells, allowing the nucleus to precipitate on the bottom of the tube to not alter hemoglobin results[34,35]. Red blood cell indices (mean corpuscular volume -MCV-, mean corpuscular hemoglobin -MCH- and mean corupuscular hemoglobin concentration -MCHC-) were calculated.

A paired t tests for treatments was performed and the differences were tested for normality with Shapiro-Wilk test. The values that didn't present a normal distribution range were compared using Wilcoxon paired test. The samples that presented any alteration (hemolysis or lypemic) suitable to interfere in the biochemistry results were excluded. Significant differences were considered with p-value <0.05. Reference ranges were calculated using means and two standard deviations with those values that presented a normality distribution using Shapiro-Wilk test; and between 2.28% to 97.73% quantiles, for values with no normal distribution. Statistical analysis was performed using the free R Core Team (2015) software[36].

3 Results

Timing of procedures was not significantly different between groups. Ambient temperatures were similar during all the experiment (Table1).

TABLE 1 - Ambient temperature (ºC)and time (in minutes)of procedures for control (SG) and midazolam treated (MG) group,difference between groups (Dif.), probability values and descriptive statistics for observed values (mean ± standard deviation - SD, range andlower and upper limits)

Ambient temperature and time of procedures	**Evaluation between groups**						**Observed values**		
	Means		**Dif.**	**p1**	**p2**	**p3**	**Mean ± SD**	**Lower limit**	**Upper limit**
	SG	**MG**							
Ambient temperature (ºC)	22.4	23.6	-1.2	0.152	0.381	-	22.96 ±1.73	20.3	26
Chasing time	1.1	1.5	-0.4	0.547	0.209	-	1.29±1.65	0	6.5
Time with the hood on	9.9	9.6	0.3	0.869	0.936	-	10.54±6.96	3	26.5
Time until treatment application	2.1	2.9	-0.9	0.330	0.320	-	2.50±1.45	1	6.5
Time in restraint cage	12.3	12.1	0.2	0.903	0.655	-	12.18±6.56	1	20.5
Duration of physical exam	5.7	5.9	-0.1	0.873	0.021	0.661	5.79±2.21	2.5	10
Duration of total restraint	2.9	3.2	-0.4	0.772	0.227	-	3.04±1.50	1	6.5
Duration of blood collection	1.6	1.4	0.2	0.791	<0.001	0.440	1.46±1.46	0	6
Total time of procedures	21.1	23.5	-2.4	0.243	0.052	0.382	22.29±3.36	17	30

p1 - p-value for paired t-test; p2 - p-value for Shapiro-Wilk normality test; p3 - p-value for Wilcoxon paired test

It was observed that midazolam application in some birds caused impaired movements and agitation rather than relaxation. Those animals had difficulties in maintaining posture. In others, it caused sedation providing an easy access for the physical exam. In one case, during the pilot study for adjusting the dosages, a bird allowed blood collection from the jugular vein without any restraint besides the hook. Another animal presented a marked stress response during saline application, manifested by hyperventilation and violent struggling within the contention box. In this individual the procedure was aborted, the animal presented ataxia and laid down, hours afterwards the animal appeared recovered and feeding. The animal was only restrained again after a week and the procedure occurred without incidences. The data of this contention were included in the study.

The only parameter that presented significant (p=0.009) differences between treatments was respiratory rate, being higher when midazolam was applied.

Reference ranges for all parameters assessed are presented in Tables 2, 3 and 4. Descriptive statistics for observed values were calculated with the mean of both collections (saline and midazolam) for each animal. For RR only the values of saline treatment were considered.

TABLE 2 - Meansfor weight, respiratory rate, heart rate and cloacal temperature from greater rheas in control group (SG) and treated midazolam group (MG), difference between groups (Dif.), provability values; and descriptive statistics for observed values (mean, standard deviation - SD, range and lower and upper limits)

Physiological parameters	**Evaluation between groups** (n indicated in each parameter)					**Observed values** (n=14)				
	Means		**Dif.**	**p1**	**p2**	**Mean**	**SD**	**Reference range**	**Lower limit**	**Upper limit**
	SG	**MG**								
Weight Kg (n=14)	-	-	-	-	-	29.99	3.39	23.20-36.78	23.30	38.30
***Respiratory rate** (n=13)	52	72	-18	0.009	0.967	52	19	14-90	20	84
Heart rate (n=14)	105	118	-13	0.364	0.991	112	38	37-187	59	208
T[a] ºC(n=14)	39.4	39.8	-0.4	0.204	0.217	39.6	0.51	38.6-40.6	38.35	40.45

p1 - p-value for paired t-test; p2 - p-value for Shapiro-Wilk normality test
For those parameters that presented statistical ($p<0.05$) difference between treatments*, in descriptive statistics calculi, only values from control group were used

TABLE 3 - Hematology parameters for greater rheasincontrol group (SG) and treated midazolam group (MG), difference between groups (Dif.), provability values; and descriptive statistics for observed values (mean, standard deviation - SD, range and lower and upper limits) in both groups, n=14

Hematology parameters	Evaluation between groups						Observed values				
	Means		Dif.	p1	p2	p3	Mean	SD	Reference range	Lower limit	Upper limit
	SG	MG									
RBC $10^6/mm^3$	2.00	2.05	-0.05	0.691	0.341	-	2.03	0.23	1.56-2.49	1.60	2.41
PCV %	43.8	44.4	-0.40	0.807	0.913	-	44.36	3.84	36.69-52.03	37	50
Hemoglobin g/dL	12.17	12.2	0.06	0.913	0.031	0.507	12.23	1.14	11.20 -14.55	11.18	15.06
MCV fl	224.3	217.6	7.08	0.569	0.726	-	220.9	25.2	170.48 - 271.23	178	273
MCH pg	62.0	60.7	1.69	0.739	0.721	-	61.3	7.9	45.51 - 77.06	51	82
MCHC %	27.9	28.2	-0.15	0.949	<0.001	0.309	28.2	3.6	25.00 - 36.12	25	37
Leucocytes $10^3/mm^3$	9.91	8.39	1.82	0.211	0.389	-	9.23	3.42	2.37-16.08	4.75	18.00
Heterophils $/mm^3$	5674	4573	1124	0.222	0.771	-	5095	2308	2088-10316	1695	11399
Lymphocytes $/mm^3$	3215	3069	400	0.472	0.433	-	3255	1331	1990-6082	1935	6370
Monocytes $/mm^3$	581	470	136	0.153	0.443	-	534	247	261- 1075	251	1186
Basophils $/mm^3$	186	136	51	0.331	0.347	-	160.07	139.69	36-490	23	613
Eosinophils $/mm^3$	242	139	94	0.044	0.036	0.168	183	144	30-495	25	509
H/L ratio	1.84	1.72	0.04	0.884	0.170	-	1.74	0.74	0.26 - 3.22	0.77	2.93
Thrombocytes $10^4/mm^3$	2.27	2.05	0.33	0.266	0.44	-	2.20	0.59	1.01-3.39	1.32	3.45

p1 - p-value for paired t-test; p2 - p-value for Shapiro-Wilk normality test; p3 - p-value for Wilcoxon paired test

TABLE 4 - Biochemistry parameters for greater rheas in control group (SG) and treated midazolam group (MG), difference between groups (Dif.), provability values; and descriptive statistics for observed values (mean, standard deviation - SD, range and lower and upper limits) in both groups

Biochemistry parameters	**Evaluation between groups** (n indicated in each parameter)						**Observed values** (n=14)				
	Means		**Dif.**	**p1**	**p2**	**p3**	**Mean**	**SD**	**Reference range**	**Lower limit**	**Upper limit**
	SG	**MG**									
ALP UI/L (n=14)	23.27	23.42	-4.79	0.323	0.595	-	23.51	17.38	2.63-56.85	2.12	59.24
ALT UI/L (n=13)	3.32	2.37	1.05	0.431	0.010	0.176	2.86	1.32	0.22-5.49	1.29	6.38
AST UI/L (n=14)	59.70	74.98	-8.79	0.264	0.736	-	69.94	21.21	48.59-114.78	48.1	125.7
Cholesterolmmol/L (n=14)	3.55	4.06	-0.23	0.378	0.915	-	4.15	1.63	2.81-8.09	2.78	8.93
CK UI/L (n=14)	641.8	568.5	104.0	0.530	0.270	-	619.5	232.8	153.9 -1085.2	316.7	1197
Creatinine µmol/L (n=14)	12.38	17.68	-5.30	0.117	0.999	-	15.56	5.30	5.30-26.52	4.42	24.75
Glucose mmol/L (n=9)	12.89	12.15	0.74	0.271	0.998	-	12.52	1.22	10.07-14.97	11.15	15.63
HDL mmol/L (n=14)	1.04	1.02	0.04	0.311	0.992	-	1.04	0.21	0.61-1.47	0.72	1.41
Lactate mmol/L (n=14)	8.66	9.48	-0.12	0.968	0.650	-	9.42	4.6	0.22-18.63	1.66	18.51
LDH UI/L (n=14)	2078.6	2769	-623.3	0.199	0.778	-	2725.3	1502.6	1428.7 -5571.8	1413	5779
Phosphorus mmol/L (n=13)	1.87	2.07	-0.35	0.096	0.074	0.131	1.97	0.37	1.23-2.71	1.28	2.50
Total plasma protein g/L (n=13)	68.7	72.8	-4.2	0.254	0.673	-	70.5	6.6	57.3-83.7	61.1	81.2
Triglicerydes g/L (n=11)	1.86	2.24	0.21	0.399	0.037	0.492	2.28	1.98	1.01-7.11	0.97	8.85
Urea mmol/L (n=14)	0.47	0.84	-0.35	0.164	0.192	-	0.64	0.46	0.04-1.53	0	1.58
Uric acid mmol/L (n=14)	0.48	0.37	0.08	0.114	0.134	-	0.4	0.11	0.16-0.54	0.09	0.56
Na(mmol/L) (n=14)	145.42	148.46	-0.04	0.988	0.474	-	148.45	11.74	140.56-176.98	139.70	187.60
K(mmol/L) (n=14)	4.60	4.63	0.09	0.753	0.700	-	4.67	0.62	3.44-5.91	3.81	6.21
Cai (mmol/L) (n=14)	1.133	1.116	0.011	0.790	0.872	-	1.12	0.07	0.99-1.25	1.02	1.23
Cl (mmol/L) (n=14)	105.52	108.89	-1	0.638	0.676	-	108.42	9.37	102.97-131.35	102.95	139.70

p1 - p-value for paired t-test; p2 - p-value for Shapiro-Wilk normality test; p3 - p-value for Wilcoxon paired test

4 Discussion

As far as we know, this is the first time that just sedation (without any further anesthesia) has been attempted in greater rheas. Avariety of anesthesia protocols have been used for restraint and management of ratites[15,37,38,39], but a deep level of unconsciousness was intended, this associated with higher probabilities of anesthesia related complications[40].

In the present study no statistical differences for biochemical and hematological parameters between treatments were detected. Restraint stress response produces changes in biochemical parameters such as elevations in CK, ALT, AST and LDH activities; and elevations in blood glucose, amino acids, fatty acids and lactate[8,24-26,41]. Hematological changes are also detected; a leukocytosis with heterophilia, lymphopenia and eosinopenia are common due to stress[26]. However, this response can vary within species and calculated heterophil/lymphocyte (H/L) ratio is considered a better stress indicator[27,28]. Restraint also is associated with fever, tachycardia, and increased RR[29-31]. All those changes are the result of increased energy demand, increased metabolism, high muscular anaerobic activity and cell lysis. Hematological and biochemical parameters have been used in assessing the effects of stress transport in emus. In those birds, six hours transport produced increases in H/L ratio, blood glucose, creatinine concentrations, CK, ALT and AST enzyme activities and decreases in triglycerides[42], as those last are the main energy resource in birds[43].The lack of differences observed in the present study could reflect no effect onstress response with midazolam sedation.

Nevertheless, it could be possible that time between the stressful event and the blood collection was too short to display changes in measured parameters. In bar-tailed godwits from New Zealand presenting CM, peak elevations in muscle enzyme activities occurred 24h post capture[14]. Elevations in enzyme activities seem to be correlated to restraint lasting time[44,45]. Changes in H/L ratios were detected within 30 min after capture in male great tits[27]. In a similar experiment with blue-and-gold macaws, significant differences in lactate concentrations between treatments were detected (not published); in passerines, changes in pH and blood gas values were also reported (time between capture and blood collection was approximately 9-12 min)[24]. This suggests that those parameters experience rapid and detectable changes within minutes post-stressful event. Responses to stress could vary between species, but our results suggest that treatment with midazolam didn't produce any differences in capture stress. Even though lactate values didn't changed between treatments, blood gas analysis could have been useful in detecting changes, as their variations

due to metabolic alterations are fast[46]. The lack of a portable blood gas analyzer and the delay that would take between sampling and processing in the lab precluded the analysis.

Corticosterone is the major glucocorticoid in greater rheas[47]. Their levels after capture in birds rise quickly[48] and their analysis could have been useful in the present study for determining the amount of stress produced by restraint.

Hematological and biochemical values can vary not only among species but also within speciesdepending on geographic regions[49,50].Values presented in this study can be used as a reference range for adult greater rheas in captivity conditions in Cerrado Biome out of breeding season. Although considerable information about reference ranges for certain ratites is available (mainly about ostriches and emus) little is been published for greater rheas[51-53], besides, the comparison between different ratite species is not justified because of their varied phylogeny. Roughly, when compared with published values our hematological and biochemical results are within ranges reported previously. Hemoglobin values in the present study presented a narrower range than reported by Fortes[51]. The technique used was identical and this difference could be attributed to a farming origin of individuals. Lymphocyte and basophil counts were higher in the present experiment and this also can be due to different environments. The most noticeable differences were cholesterol, total protein, LDH and CK activities, all higher than previously published values from a wild population in Argentina[53]. This could be attributed to differences in lifestyle (wild and captivity) and also different sampling periods, thus wild birds were handled at night. Circadian variations in biochemical parameters and in corticosterone plasma concentrations have been described[54,55].

Another important thing to consider when comparing values is that most of biochemistry tests realized in this experiment were performed in heparinized plasma (except for glucose and lactate) rather than serum. For most of the tests, manufacturers' instructions allow both, serum and plasma, except for total protein, triglycerides and LDH for which serum rather than heparinized plasma is indicated. The main difference between serum and total plasma protein concentrations is that the second includes fibrinogen and for that, higher values are expected. Heparin enhances the activation of lipoprotein lipase, producing decreases in triglycerides values[56], but even after a few months of storage, triglyceride values in this study were similar or higher when compared with literature[53,57], however this cannot exclude the possibility of false lowered values.

Notably high LDH activities were reported in this study. Falsely increased LDH concentrations have been associated with erythrocytes and platelet contamination, which contain high concentrations of this enzyme[58].LDH results are highly affected by hemolisis[59],

in fact, activities of this enzyme are used as biomarkers for syndromes that produce hemolysis in medicine[60]. In the present study, even not visually perceptible, certain amount of hemolysis could be present in plasma sampled, elevating LDH activities. Measurement of hemoglobin plasma concentration could have been useful for elucidate this interference.

Contrary as expected, midazolam treatment at 1 mg/kg dose didn't provide adequate sedation in greater rheas. It was reported that, when receiving midazolam, some animals presented ataxia, producing struggling in some of them when trying to maintain a standing position. It was also observed that the reaction depended upon each individual, as such, some stayed calmer than when saline solution was administered. The only significant difference between treatments was RR, being higher with midazolam, which could be explained by a higher excitement produced in some animals, leading to hyperventilation[61]. In horses, weakness and ataxia produced by benzodiazepines disable their use in adults; however, they are an extremely effective sedation alternativein young foals, which become recumbent so that ataxia is not a problem[62].

Differentto what has been observed in the present study, in other species of birds, midazolam has proved a good sedation alternative[16,17,21,23,63,64].However, it is important to keep in mind that the phylogeny between ratites and other bird species might be distant. Dosageadjustements might differ as much as between a horse and a cat, for example[65]. In Canada geese, the use of 1 mg/kg midazolam resulted inadequate for sedation but with 2 mg/kg a moderate sedation was achieved producing an increase of RR at 10-30 min post injection, which didn't affect blood pressure, heart rate or temperature[66]. Inquail, IM 4mg/kg or 6mg/kg midazolam induced sedation with a 10 minutes onset of action, lasting longer with the highest dose. The need of adjusting dosages depending on the degree of animal excitement presented previous to sedation has been suggested[15]. Higher doses have been used successfully in birds for sedation purposes, however, usually with an intranasal administration[16,17,23,63,64]. In pigeons this administration route demonstrated to provide quicker recovery times than an intramuscular route[67].

The dose used in the present experiment was established after a pilot test. At first, the intention was to administer the midazolam via an intranasal route. The need for a higher concentrated drug and the impossibility of commercial presentation availability in Brazil, led to various attempts of concentrating the 5mg/ml presentation with a rotary evaporator (IKA® 10, Staufen, Germany). The administration of 1 mg/kg IN midazolam in one animal produced a rapid and optimal sedation that even allowed jugular blood collection without any further restraint than a hook. However, the difficulty for obtaining a stable concentration drug

prevented this administration route. Finally, the IM route with a dosage of 1 mg/kg was adopted. Higher dosages were also limited because of volume administration. The biceps brachii muscle was chosen as application local in order to avoid the renal portal system.

The variability of responses presented within birds in this study could be produced by irregular drug absorption with IM route. In ostriches the use of IV midazolam injection in a dose of 0.3 mg/kg produced sedation; increasing the dose to 1 mg/kg induced hypnosis (loss of palpebral and oral reflexes allowing endotracheal intubation) and recumbence for a brief period (5 min). The authors hypothesize that a constant rate infusion would be necessary to maintain hypnosis[38]. In this case the IV route provably resulted in higher plasma concentrations when compared to IM administration.

A possible alternative for the present study could have been the IN route administration.IN route is easier and less invasive, a rapid diffusion of the drug into the circulatory system is ensured by the highly irrigated epithelium of the nasal cavity, also avoiding first pass methabolism[68]. When compared to intra venous administration in humans, IN midazolam presented high bioavailability (82%)[69]and this application routewas considered suitable for use in situations where a fast and non-invasively route is required[70]. Differences in therapeutic responses between benzodiazepine administration routes have been described in human patients, and specifically, the IM route producing less predictable and later response than intravenous or oral administration[71]. In birds, further studies should be done to supply the lack of information about midazolam pharmacokinetics.

In the present study a high individual variation in response to restraintwas reported. In rheas, stress responses might differ between sexes[72] and among individuals[73,74],thus supporting the need of dose adjustments. Also, the response in corticosterone plasma levels after an ACTH challenge in this species was noticeable when compared to increases reported for other avian species. This could suggest a major stress response in this specie, which is consistent with the antipredatory strategy that performs fast running, and therefore, a great amount of energy resources is needed[47]. Considering that, higher midazolam doses might have been more effective in this species.

Some birds presented sings of agitation after midazolam application, this being attributed either to a drug mediated response or to discomfort due to difficulty in maintaining equilibrium. In human patients benzodiazepine sedation occasionally may elicit signs of agitation, aggression and hostility, a phenomenon known as paradoxical reactions[75]. The use of an analgesic together with midazolam sedation seemed to reduce the incidence of paradoxical reactions in patients' ongoing endoscopic procedures[76]. Higher dosages, high

potencies and short half live benzodiazepines seem to be correlated with those reactions[77]. In dogs, high incidences of hyperactivity and agitation have been reported after midazolam administration[78], however a clear explanation of this phenomenon has not been elucidated.

The use of midazolam in combination with other analgesics or anesthetics could be an alternative for preventing the controversial effects reported here. In adult horses, benzodiazepines may be used as part of anaesthetic combinations[62]. In pigeons the use of midazolam and ketamine for anesthesia showed good results[67]. Midazolam appeared to have a beneficial effect in limiting excitement and struggling during recovery after ketamine anesthesia in ostriches[38]. Also, if given as a pre-anesthetic sedation, even with no detectable effects on birds' behavior, they appeared to struggle less during restraint and also effects were seen during recovery, which was slow and smooth[65].

Midazolam, even though not effective at 1 mg/kg IM for sedation, produces minimal adverse effects. Eventually, an intranasal use of midazolam for sedation in greater rheas could be an alternative, but higher concentration presentations are needed. It might be easier in animals that were conditioned[38] and reared in captivity. Also higher dosages could be attempted.

In conclusion, IM midazolam at 1 mg/kg dose didn't produce differences in stress response to capture when compared with a no sedation restraint protocol.

No cases of CM were reported during this experiment and the only animal that presented the most exacerbated stress response recovered rapidly and is healthy until present data (six months later).

Hematological and biochemical values presented here contribute to knowledge and information about this specie, which, currently, is sparse.

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CAPITULO 4 CONSIDERAÇÕES FINAIS

Na prática, durante o manejo de animais silvestres, as técnicas de sedação são ainda pouco utilizadas. Estas ganham importância em animais de vida livre, que quando manipulados apresentam uma resposta estressante muito maior do que aqueles da mesma espécie já condicionados ou acostumados a certas práticas de manejo. Em centros de reabilitação, nos quais os animais encontram-se debilitados ou feridos e ainda sob estresse crônico em decorrência do cativeiro, as técnicas que visam minimizar o impacto da contenção, necessária para múltiplos procedimentos, são essenciais. O fato de diminuir o estresse nos momentos da manipulação dos animais, não somente traz benefícios durante a realização do procedimento, mas também pode ajudar no processo de reabilitação, assegurando melhores condições para superar as dificuldades iniciais do momento da liberação.

O presente trabalho demonstrou que o midazolam intranasal na dose de 7 a 11 mg/kg aplicado em forma de spray é efetivo na diminuição do estresse por contenção em araras canindé. O fato de não dispor de uma formulação mais concentrada do fármaco dificultou em certo grau a administração, pois foi necessário espraiar múltiplas vezes em cada narina para atingir o volume. Neste aspecto, a disponibilidade de concentrações maiores do fármaco facilitaria a aplicação, o que possibilitaria também o seu uso nas emas, nas quais o volume para atingir a dose necessária foi muito grande, impedindo a administração intranasal. As tentativas de concentração do fármaco tiveram sucesso em alguma ocasião, mas a repetibiliade da técnica não foi possível. O fato de precisar de grandes volumes para obter a quantidade necessária de midazolam concentrado, assim como a obtenção de concentrações diferentes a cada tentativa foram os fatores que impediram a padronização.

A administração intranasal nas emas provavelmente teria evitado as variações observadas em resposta ao midazolam, pois possivelmente, estas foram o resultado de absorção irregular do fármaco por via IM.

Em medicina, novas formulações para o midazolam intranasal estão sendo investigadas. Estas visam melhorar a absorção por esta via tentando diminuir os efeitos adversos,gerando fórmulas que sejam menos irritantes. A aplicação de tais produtos tem a finalidade de tratar crises convulsivas em humanos. Portanto, a rapidez dos efeitos é fundamental. Isto cria expectativas promissoras também no campo do trabalho com animais silvestres. A facilidade de aplicação intranasal permite que possa ser usada em multiplicidade de situações diminuindo os riscos associados a vias mais invasivas. Nas aves,em que o aceso

venoso é difícil, a aplicação IN está plenamente justificada, pois também produz rapidez de ação quando comparada a aplicação IM. No entanto, mais estudos sobre o efeito deste fármaco na mucosa nasal das aves devem ser conduzidos, assim como sobre sua farmacocinética, uma vez que éstapode variar para cada espécie de ave.

Algumas limitações devem ser consideradas. As reações de regurgitação descritas em araras e outras aves podem levar a complicações respiratórias gravíssimas. Para evitar tais riscos é fundamental respeitar um período de jejum prévio e ter ao alcance a droga antagonista. Com respeito à aplicação intranasal, a presença de secreção nasal pode dificultar a absorção. Neste experimento, a presença de secreção nasal foi observada em algumas aves, porém não pareceu ter exercido efeito sobre a sedação.

É importante salientar que a sedação produzida pelo midazolam parece diminuir em caso de estimulação do animal. Para a sedação ser efetiva era necessário fornecer um ambiente silencioso e escuro (tipo caixa) até o fármaco surgir efeito. Depois disso, a manipulação tinha que ser cuidadosa e os estímulos minimizados. Na hora da punção para a colheita de sangue a sedação perdia efeito fazendo necessária a contenção do animal. Mesmo assim, a contenção era mais tranquila e o animal seguia sem apresentar vocalização. Em certa ocasião, a mesma técnica de sedação foi utilizada para realização de raios X em duas araras do centro de reabilitação com excelentes resultados. O tempo de sedação foi longo o suficiente para realizar vários posicionamentos sem necessidade de conter o animal. Deste modo, parece que na ausência de estímulo nociceptivo a sedação com midazolam apresenta-se mais eficiente e permitindo a manipulação da ave sem necessidade de contenção física. Uma alternativa para conseguir melhor sedação na hora da punção venosa ou de qualquer outro procedimento com potencial nociceptivo seria a administração conjunta de um analgésico. No caso das aves o butorfanol parece ser o mais eficiente e tal combinação já foi testada com sucesso. Mais estudos a respeito disso devem ser conduzidos.

No caso das emas, depois de contemplar a variabilidade da resposta ao midazolam em função do indivíduo durante o estudo piloto, a possibilidade de associação de fármacos foi considerada. Porém, ponderou-se que seria mais correto estudar os efeitos exculsivamente do midazolam,sem outras interações que tornariam mais difícil o discernimento sobre o efeito de cada fármaco.

O brete de contenção utilizado nas emas também promoveu reações diferentes, sendo que algumas aves pareciam tolera-lo bem, enquanto outras debatiam-se muito, sendo necessário retirá-las do brete deixando a ave se locomover livremente na sala. Durante os procedimentos piloto, nos quais não foi utilizado o brete, após a administração do fármaco e

mesmo com o capuz na cabeça, o animal seguia se movimentando, chocando-se contra as paredes e sacudindo a cabeça para tentar tirar o capuz. O objetivo da utilização do brete foi precisamente fornecer um ambiente escuro onde o animal ficasse quieto até o fármaco surgir efeito. Em alguns indivíduos pareceu ser efetivo e em outros produziu a reação contrária, podendo assim impedir que o fármaco fizesse efeito. Para padronizar os efeitos do brete, tentou-se proceder da mesma maneira durante os dois tratamentos, sendo que quando um animal tinha apresentado intolerância ao brete no primeiro período, no segundo tentava-se mantê-lo dentro do brete pelo mesmo tempo que tinha sido mantido na primeira vez. Curiosamente, durante o segundo período alguns animais que tinham apresentado intolerância ao brete na primeira ocasião, se apresentaram mais tranquilos. Mesmo assim foram mantidos no brete pelo mesmo período que a vez anterior. Isto demonstra certo condicionamento dos animais que ao longo do experimento foram apresentando mais tolerância ao manejo. Tal efeito pode ter mascarado possíveis diferenças entre tratamentos neste experimento.

Nas emas, uma alternativa para melhorar a sedação teria sido aumentar as doses, porém, como já comentado, o volume foi um fator limitante. Por serem animais maiores e portanto, com taxas metabólicas menores, é de se esperar que doses mais baixas sejam preconizadas. Além disso, as reações de ataxia apresentadas em alguns animais pareciam desencadear excitação no intento de manter a postura. Possivelmente, maiores doses não teriam evitado tais reações, pois as emas apresentam relutância para deitar. Pode ser que o fornecimento de uma estrutura que ajudasse a mantê-las em posição pudesse melhorar tais efeitos, permitindo atingir a sedação. De qualquer maneira, os protocolos de sedação em emas dificilmenteserão efetivos em animais de vida livre, sem nenhum manejo e contato com humanos, nos quais a resposta ao estresse por contenção deverá ser mais exacerbada.

As emas e as araras têm propensões diferentes a apresentar quadros de miopatia por captura, sendo as emas muito mais susceptíveis. De fato, não existe na literatura nenhum caso de miopatia por captura em espécies de araras. Este foi um dos critérios para a seleção de ambas as espécies para o desenvolvimento deste projeto, pois provavelmente as duas apresentam diferenças na resposta ao estresse por contenção, sendo mais exacerbada nas emas. A dificuldade na sedação das emas observada no presente estudo confirma tais suspeitas. Assim, os diferentes graus de estresse em resposta à contenção em função das espécies e, em consequência, o risco de complicações derivadas, devem ser sempre ponderados na hora da manipulação de aves. Alternativas para minimizar os riscos dascomplicações da contenção, principalmente nas espécies que apresentam uma maior propensão a miopatiapor captura, devem ser investigadas.

Outra vantagem do midazolam no manejo de animais silvestres são seus efeitos amnésicos. Tais efeitos não foram estudados em aves, mas se confirmados, seriam muito positivos por impedir associações que poderiam dificultar manejos repetidos. Também a estimulação do apetite relatada em alguns estudos após a aplicação do midazolam é positiva no caso de animais em recuperação e debilitados.

Neste estudo, evidencia-se a importância da mensuração das concentrações de lactato para caracterizaro estresse e a acidose metabólica resultanteda contenção das aves. Mesmo nos estudos que não têm por objetivo avaliar o estresse originado pela contenção, a gasometria aliada a medição do lactato é fundamental. As menores concentrações de lactato das araras sedadas com midazolam é a prova que este contribui para diminuir o estresse por contenção nesta espécie. O fato deste parâmetro experimentar rápida elevação também permite visualizar os efeitos da contenção emcurto prazo.

Com respeito aos demais parâmetros mensurados, provavelmente, se as amostras de sangue tivessem sido colhidas mais tardiamente ao momento da contenção, teriam sido detectadas alterações. Outra alternativa teria sido amostras seriadas, porém seria difícil evitar o efeito provocado por contenções seriadas.

Uma dificuldade encontrada durante o estudo foi a falta de informação com respeito aos valores de referência para estas espécies. Muitas vezes, os valores disponíveis são de populações mantidas em condições geográficas diferentes às do cerrado podendo apresentar variações. A necessidade de criar bancos de dados considerando as populações locais é fundamental no monitoramento da situação da conservação de tais espécies no seu hábitat natural. Tais informações também podem ajudar na preservação do bioma cerrado, cada vez mais ameaçado, uma vez que as aves podem ser utilizadas como biomarcadores.

Em conclusão,podemos afirmar que o midazolam INapresentou-se como uma boa alternativa na sedação de araras canindé, sendo seguro e efetivo na diminuição do estresse por contenção nesta espécie, apresentando mínimos efeitos adversos. Já nas emas, a sedação com midazolam apresentou resultados variáveis evidenciando a necessidade de mais estudos com respeito a vias de absorção e possibilidade de associações com outros fármacos.

ANEXO A - APROVAÇÃO PELO CEUA

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

Goiânia, 05 de agosto de2014.

PARECER CONSUBSTANCIADO REFERENTE AO ATENDIMENTO DE PENDÊNCIADO PROTOCOLO Nº. 075/13

I. IDENTIFICAÇÃO:

1. **Título do projeto**: Efeito do midazolam no estresse por contenção em aves silvestres
2. **Pesquisador Responsável**: Laura Garcia Silva
3. **Unidade/Órgão do pesquisador:** Escola de Veterinária e Zootecnia da UFG.
4. **Pesquisadores Participantes:** Maria Clorinda Soares Fioravanti, Juan Carlos Duque Moreno, Celina TieNishimori Duque, Talita Dayane Pereira e Silva, Helton Freires Oliveira.
5. **Unidade onde será realizado:** Escola de Veterinária e Zootecnia da UFG.
6. **Data de apresentação do protocolo a CEUA:** 09.12.2013
7. **Data de Atendimento das Pendências:** 14.07.2014

II - Parecer da CEUA:

Informamos que a *Comissão de Ética no Uso de Animais/CEUA* da Universidade Federal de Goiás, após análise das adequações solicitadas, **Aprovou** o projeto acima referido, o qual foi considerado em acordo com os princípios éticos vigentes.

Reiteramos a importância deste Parecer Consubstanciado, e lembramos que o(a) pesquisador(a) responsável deverá encaminhar à CEUA-PRPPG-UFG o Relatório Final baseado na conclusão do estudo e na incidência de publicações decorrentes deste, de acordo com o disposto na Lei nº. 11.794 de 08/10/2008, e Resolução Normativa nº. 01, de 09/07/2010 do Conselho Nacional de Controle de Experimentação Animal-CONCEA. O prazo para entrega do Relatório é de até 30 dias após o encerramento da pesquisa, prevista para conclusão em Abril de 2015.

III - Data da reunião: 14.07.2014

RENATA MAZARO:12343522812

Assinado de forma digital por RENATA MAZARO:12343522812
DN: c=BR, o=ICP-Brasil, ou=Autoridade Certificadora Raiz Brasileira v2, ou=AC SOLUTI, ou=AC SOLUTI Multipla, ou=Certificado PF A3, cn=RENATA MAZARO:12343522812
Dados: 2014.08.05 23:47:19 -03'00'

Dra. Renata Mazaro e Costa
Coordenadora da CEUA/PRPI/UFG

ANEXO B - AUTORIZAÇÃO DO SISBIO

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 42088-1	Data da Emissão: 09/12/2013 15:00	Data para Revalidação*: 08/01/2015

* De acordo com o art. 33 da IN 154/2009, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Maria Clorinda Soares Fioravanti	CPF: 370.994.261-68
Título do Projeto: EFEITO DO MIDAZOLAM NO ESTRESSE POR CONTENÇÃO EM AVES SILVESTRES	
Nome da Instituição : UNIVERSIDADE FEDERAL DE GOIAS	CNPJ: 01.567.601/0001-43

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Treinamento	12/2013	01/2014
2	Revisao bibliográfica	12/2013	02/2015
3	Primera colheita e processamento de amostras	02/2014	03/2014
4	Processamento das amostras	02/2014	07/2014
5	Segunda colheita e processamento de amostras	04/2014	04/2014
6	Análise de resultados	05/2014	08/2014
7	Redaçao da dissertaçao	10/2014	02/2015
8	Defesa e publicaçao	03/2015	03/2015

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa IBAMA nº 154/2007 ou na Instrução Normativa ICMBio nº 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio e o material biológico coletado apreendido nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	O membro da equipe estrangeiro Juan Carlos Duque Moreno é portador de visto permanente no Brasil; o membro estrangeiro Laura García Vila participa de Programa de bolsas ou auxílio à pesquisa patrocinado pela CAPES, estando portanto, dispensados de autorização do Ministério da Ciência, Tecnologia e Inovação.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Juan Carlos Duque Moreno	Comitê de orientaçao	219.612.488-59	V252252-4 CGPI/DIREX-SP	Estrangeira
2	Laura García Vila	Pesquisador	701.842.481-08	V750644-0 DPF-GO	Estrangeira
3	CELINA TIE NISHIMORI DUQUE	Comitê de orientaçao	251.961.138-35	20110071-X SSP-SP	Brasileira

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Dados do titular

Nome: Maria Clorinda Soares Fioravanti	CPF: 370.994.261-68
Título do Projeto: EFEITO DO MIDAZOLAM NO ESTRESSE POR CONTENÇÃO EM AVES SILVESTRES	
Nome da Instituição : UNIVERSIDADE FEDERAL DE GOIAS	CNPJ: 01.567.601/0001-43

4	Talita Dayane Pereira e Silva	Auxiliar	012.246.571-79	4636952 DGPC-GO	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	GOIANIA	GO	Zoológico Municipal de Goiânia	Fora de UC Federal
2	GOIANIA	GO	Centro de Triagem de Animais Silvestres	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas ex situ	Ara ararauna, Rhea americana

Material e métodos

1	Amostras biológicas (Aves)	Sangue
2	Método de captura/coleta (Aves)	Puçá, Outros métodos de captura/coleta(Gancho)

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	UNIVERSIDADE FEDERAL DE GOIAS	

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº154/2007, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

* Identificar o espécime no nível taxonômico possível.

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº154/2007. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 76359833

ANEXO C - VALORES DE REFERÊNCIA PARA ARARA CANINDÉ

Valores hematológicos *Ara ararauna*

Parâmetro	Valor médio	Rango	N	Autor
RBC $10^6/mm^3$	2,56	2,30 - 2,80	29	*Gomes et al.*
	-	2,70 - 3,50	12	*Hawkey & Samour*
	-	2,66 - 3,83	20	*Bonello et al.*
	3,24 ± 0,50	2,11 - 4,10	35	*Polo et al.*
	2,21 ± 0,45	1,76 - 2,66	13	*Santos*
Hematócrito %	34,50	32,1-36,8	29	*Gomes et al.*
	46 ± 3	41-51	12	*Hawkey & Samour*
	44,60 ± 4,60	31,5-51,8	35	*Polo et al.*
	47 ± 4	43-51	13	*Santos*
Hb g/ dl	14,90	13,80-16	29	*Gomes et al.*
	16,80 ± 1,20	14,80-18,90	12	*Hawkey & Samour*
	-	11,30-15,31	20	*Bonello et al.*
	14,40 ± 1,20	11,70-17	35	*Polo et al.*
	15,89 ± 1,55	14,34-17,44	13	*Santos*
VCM μ^3	138,5	125,9-151	29	*Gomes et al.*
	146 ± 7	132-157	12	*Hawkey & Samour*
	-	130,55-171,43	20	*Bonello et al.*
	141 ± 21,4	102,4-199,1	35	*Polo et al.*
	221,69 ± 36,37	185-258	13	*Santos*
HCM pg	59,60	54,9-64,3	29	*Gomes et al.*
	52,30 ± 2,20	49,4-56,4	12	*Hawkey & Samour*
	46,4 ± 6,5	34,6-61,9	35	*Polo et al.*
	72,53 ± 11,08	61,0-83,0	13	*Santos*
CHCM %	43,60	41,10-46,00	29	*Gomes et al.*
	36,60± 1,70	34,70-39,80	12	*Hawkey & Samour*
		26,44-32,58	20	*Bonello et al.*
	32,70± 3,40	28,10-43,50	35	*Polo et al.*
	33,02 ± 1,63	31,00-34,00	13	*Santos*
Trombócitos $10^4/mm^3$	0,98	0,84 - 1,12	5	*Gomes et al.*
	2,10 ± 0,60	1,10-3,40	12	*Hawkey & Samour*
Leucócitos $/mm^3$	4640	2900-6300	29	*Gomes et al.*
	4500 ± 3080	1400-7500	13	*Santos*
	8500 ± 3600	4500-15400	12	*Hawkey & Samour*
	16600 ± 9000	1700-36000	35	*Polo et al.*

	%	**$/mm^3$**	**%**	**$/mm^3$**		
Heterófilos	-	3116,8	-	2025,9-4207,6	29	*Gomes et al.*
	-	5000 ± 1500	49-71	2300-8000	12	*Hawkey & Samour*
	37,2 ± 18,3		12,8-60	-	35	*Polo et al.*
	73 ± 14,49	3680 ± 3046,72	58-87	633-6726	13	*Santos*

	-	1188,70	-	772,9-1604,5	29	*Gomes et al.*
Linfócitos	-	2000 ± 600	18-53	900-3300	12	*Hawkey & Samour*
	20 ±14,06	1118 ± 1020,07	5-34	97-2138	13	*Santos*
	60 ± 17,60	-	35,5-84,4	-	35	*Polo et al.*
	-	180,10	-	55,9-304,3	29	*Gomes et al.*
Monócitos	-	-	0-2	0-100	12	*Hawkey & Samour*
	1,30 ± 0,80	-	0-2	-	35	*Polo et al.*
	0,00 ± 0,87	0,00 ± 94,21	0-1	0-94	13	*Santos*
	-	155,40	-	79,20-231,60	29	*Gomes et al.*
Eosinófilos	-	-	0-1	-	12	*Hawkey & Samour*
	0,00 ± 0,66	0,00 ± 38,02	0-1	0-38	13	*Santos*
	0,70 ± 0,80	-	0-2	-	35	*Polo et al.*
	-	6,40	-	0-14,40	29	*Gomes et al.*
Basófilos	-	-	0-1	0-200	12	*Hawkey & Samour*
	0,30 ± 0,60	-	0-1,60	-	35	*Polo et al.*
	0	0	0	0	13	*Santos*

Valores bioquímicos *Ara ararauna*

Parâmetro	Valor médio	Rango	N	Autor
Ácido úrico (μmol/L)	-	M 112,1 - 224,2 F 141,6 - 224,2	24	*Valle et al.*
	-	171,1 - 501,5	-	*Hochleithner**
	416,3	237,9 - 600,7	-	*Samour*
	296 ± 147	113 - 629	35	*Polo et al.*
	-	109 - 231	-	*Lumeij & Overduin**
	-	112,1 - 649	-	*Fudge**
	-	147,5 - 649	-	*Cubas et al.*
Ácidos biliares (μmol/L)	-	25 - 71	-	*Lumeij & Overduin**
	-	27 - 86	-	*Fudge**
	-	6 - 35	-	*Cubas et al.*
Albumina (g/L)	-	13-15	24	*Valle et al.*
	-	11 - 24	-	*Kaneko et al.**
	-	12,4 - 31,1	-	*Capitelli & Crosta**
	-	12,5 - 30,8	80	*Cray et al.*
	22,5	19 - 26	-	*Samour*
	-	20 - 28	-	*Cubas et al.*
ALT	-	22 - 105	-	*Lumeij & Overduin**
	186	99 - 263	-	*Samour*
	8,1 ± 3,3	3,5 - 15,7	35	*Polo et al.*
	-	5 - 15	-	*Hochleithner**
	-	5 - 12	-	*Cubas et al.*
Amilase (UI/L)	-	239 - 516	-	*Fudge**
	-	276 - 594	-	*Hochleithner**
AST (UI/L)	-	111,6 - 130	24	*Valle et al.*
	67,9	61,4 - 74,5	29	*Gomes et al.*
	-	40 - 2408	-	*Kaneko et al.**
	-	58 - 206	-	*Lumeij & Overduin**
	-	64 - 168	-	*Fudge**
	247	197 - 297	-	*Samour*
	56,2 ± 19,1	33 - 105	35	*Polo et al.*
	-	45 - 125	-	*Hochleithner**
Bilirrubina total (μmol/L)	2,56	1,71 - 3,42	-	*Samour*
Cálcio (mmol/L)	-	M 2,22 - 2,4 F 2,25 - 2,58	24	*Valle et al.*
	-	1,93 - 3,73	-	*Kaneko et al.**
	-	2,1 - 2,95	-	*Fudge**
	-	1,7 - 2,48	-	*Hochleithner**
	-	2,13 - 3,25	-	*Cubas et al.*
	-	2,2 - 2,8	-	*Lumeij & Overduin**
	2,3 ± 0,4	1,7 - 3,2	35	*Polo et al.*
Cálcio iônico (mmol/L)	5,4	4,6 - 6,2	-	*Samour*
CK (UI/L)	-	M 151,6 - 200,3 F 112,5 - 160,9	24	*Valle et al.*
	86,4	65,4 - 107,3	29	*Gomes et al.*
	-	61 - 531	-	*Lumeij & Overduin**
	-	88 - 361	-	*Fudge**
	131 ± 109	35,4 - 428	35	*Polo et al.*
	-	39 - 384	-	*Hochleithner**
	-	180 - 500	-	*Cubas et al.*

Cl (mmol/L)	106,5	103 - 110	-	*Samour*
	101 ± 9,1	75 - 122	35	*Polo et al.*
	-	97 - 126	-	*Hochleithner**
Colesterol (mmol/L)	-	4,8 - 5,59	24	*Valle et al.*
	5,15	4,42 - 5,87	29	*Gomes et al.*
	-	2,49 - 6,45	-	*Fudge**
	-	2,8 - 5,18	-	*Hochleithner**
	-	2,59 - 10,1	-	*Cubas et al.*
	4,39	3,59 - 5,22	-	*Samour*
	4,2 ± 0,9	3,1 - 6,7	35	*Polo et al.*
Creatinina (µmol/L)	-	35,36 - 176,8	-	*Kaneko et al.**
	-	8,84 - 44,2	-	*Capitelli & Crosta**
	-	8,84 - 35,36	-	*Hochleithner**
	-	44,2 - 53,04	-	*Cubas et al.*
	-	20 - 59	-	*Lumeij & Overduin**
	35,3	26,52 - 44,2	-	*Samour*
	49,1 ± 15,1	26,1 - 76,8	35	*Polo et al.*
FA UI/L	-	223,3 - 362	24	*Valle et al.*
	-	10 - 239	-	*Kaneko et al.**
	371	162 - 580	-	*Samour*
	194 ± 81,9	79 - 357	35	*Polo et al.*
	-	25 - 152	-	*Hochleithner**
	-	290 - 750	-	*Cubas et al.*
Fe (µmol/L)	19,16	14,1 - 24,1	-	*Samour*
	-	14,14 - 24,17	-	*Cubas et al.*
Fibrinogênio (g/L)	1,9 ± 0,6	1 - 3,2	-	*Hawkey et al.*
Fósforo inorgânico (mmol/L)	-	1,68 - 1,97	24	*Valle et al.*
	-	0,65 - 3,36	-	*Kaneko et al.**
	-	0,65 - 3,88	-	*Capitelli & Crosta**
	0,74	0,61 - 0,83	-	*Samour*
	4,5 ± 1,1	3 - 6,2	35	*Polo et al.*
	-	1,3 - 4,8	-	*Hochleithner**
Frutosamina (mmol/L)	-	1,2 - 1,3	24	*Valle et al.*
GGT (UI/L)	-	<1 - 5	-	*Lumeij & Overduin**
	3,7 ± 5,8	0 - 18,5	35	*Polo et al.*
GLDH (UI/L)	-	<8	-	*Lumeij & Overduin**
Glicose (mmol/L)	12,76	11,89 - 13,63	29	*Gomes et al.*
	-	11,95 - 23,24	-	*Kaneko et al.**
	-	11,68 - 20,46	-	*Fudge**
	16,48 ± 0,86	15,62 - 17,34	13	*Santos*
	-	12,79 - 18,13	-	*Hochleithner**
	-	8,06 - 18,13	-	*Cubas et al.*
	17,1	15,8 - 18,4	-	*Samour*
	15,1 ± 2,1	10,1 - 19	35	*Polo et al.*
	-	12 - 17,9	-	*Lumeij & Overduin**
Globulinas (g/L)	-	8 - 33	-	*Kaneko et al.**
	25,5	21 - 30	-	*Samour*
	20	-	-	*Cubas et al.*
Globulinas alfa1 (g/L)	-	0,4 - 2,5	-	*Capitelli & Crosta**
	-	0,4 - 2,5	80	*Cray et al.*
Globulinas alfa2 (g/L)	-	0,4 - 3,1	-	*Capitelli & Crosta**
	-	0,4 - 3,1	80	*Cray et al.*
Globulinas beta (g/L)	-	1,4 - 6,2	-	*Capitelli & Crosta**
	-	3,8 - 5,4	80	*Cray et al.*

Globulinas gamma (g/L)	-	1 - 6,2	-	*Capitelli & Crosta**
	-	2,1 - 5,5	80	*Cray et al.*
K^+ (mmol/L)	-	2,2 - 10,1	-	*Kaneko et al.**
	-	1,9 - 4,1	-	*Lumeij & Overduin**
	7,4	5 - 10,4	-	*Samour*
	2,4 ± 0,8	1,7 - 4,7	35	*Polo et al.*
	-	2,1 - 4,5	-	*Hochleithner**
	-	2- 5	-	*Capitelli & Crosta**
LDH (UI/L)	233,5	193,8 - 273,2	29	*Gomes et al.*
	-	48 - 831	-	*Kaneko et al.**
	-	66 - 166	-	*Lumeij & Overduin**
	-	69 - 220	-	*Fudge**
	423,5	183 - 664	-	*Samour*
	140 ± 81,3	61,7 - 349	35	*Polo et al.*
	-	65 - 400	-	*Hochleithner**
	-	70 - 350	-	*Cubas et al.*
Mg (mmol/L)	1 ± 0,2	0,7 - 1,4	35	*Polo et al.*
Na^+ (mmol/L)	-	138 - 157	-	*Kaneko et al.**
	-	150 - 175	-	*Lumeij & Overduin**
	145,7	138 - 153	-	*Samour*
	150 ± 28,6	119 - 252	35	*Polo et al.*
	-	133 - 160	-	*Hochleithner**
	-	140 - 165	-	*Capitelli & Crosta**
Nitrogênio urinario (mmol/L)	-	0,3 - 3,3	-	*Kaneko et al.**
	2,14	0,74 - 3,57	-	*Samour*
Osmolalidade (mmol/kg)	-	319 - 378	-	*Kaneko et al.**
	319 ± 6,2	309 - 328	35	*Polo et al.*
Pre albumina (g/L)	-	5 - 11,1	-	*Kaneko et al.**
	-	0,5 - 7	-	*Capitelli & Crosta**
	-	2,5 - 8,5	80	*Cray et al.*
Proteína total (g/L)	-	35 - 38	24	*Valle et al.*
	34,3	31,4 - 37,2	29	*Gomes et al.*
	-	22 - 52	-	*Kaneko et al.**
	-	33 - 53	-	*Lumeij & Overduin**
	-	25 - 42	-	*Fudge**
	-	22 - 46	80	*Cray et al.*
	-	40 - 56	20	*Bonello et al.*
	49,5	43 - 56	-	*Samour*
	48 ± 3,4	44 - 51	13	*Santos*
	-	22 - 44	-	*Hochleithner**
	-	43 - 56	-	*Cubas et al.*
Razão albumina/globulinas	-	1,4 - 3,9	-	*Kaneko et al.**
	-	1,2 - 2,6 (com pre-albumina)	80	*Cray et al.*
	-	0,8 - 2 (sem pre albumina)	80	*Cray et al.*
	-	1,6 - 4,3	-	*Capitelli & Crosta**
	0,75	0,6 - 0,9	-	*Samour*
	-	1,6 - 4,3	-	*Cubas et al.*
Razão BUN/creatinina	8	3 - 13	-	*Samour*
Razão ureia/ácido úrico	-	5 - 28	-	*Lumeij & Overduin**
Triglicérides (g/L)	0,99	0,7 - 1,28	29	*Gomes et al.*
	-	0,67 - 1,25	-	*Hochleithner**
	1,04	0,30 - 1,55	-	*Samour*
	1,09 ± 0,64	0,36 - 2,27	35	*Polo et al.*

	-	0,835 - 1,085	24	*Valle et al.*
Uréia (mmol/L)	-	0,301 - 3,307	-	*Kaneko et al.**
	-	0,300 - 3,300	-	*Lumeij & Overduin**
	1,3 ± 0,5	0,400 - 2,100	35	*Polo et al.*
Uratos (mmol/L)	-	0,088 - 0,871	-	*Kaneko et al.**

* Valores gerais para o gênero *Ara* sp.

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ANEXO D - VALORES DE REFERÊNCIA PARA EMA

Valores hematológicos *Rhea americana*

Parâmetro	Valor médio		Rango		N	Autor
RBC	2,40		1,60 - 2,90		10	*Fortes*
$10^6/mm^3$	2,25		-		-	*Green & Blue-Maclendon*
Hb	16,30		9,40 - 26,00		10	*Fortes*
g/dl	13,40		-		-	*Green & Blue-Maclendon*
Hematócrito	39,60		30 - 45		10	*Fortes*
	41,60		-		-	*Green & Blue-Maclendon*
%	49,40		44 - 54		22	*Uhart et al.*
VCM	171		-		1	*Fortes*
μ^3	185		-		-	*Green & Blue-Maclendon*
CHCM	42,1		-		10	*Fortes*
%	32		-		-	*Green & Blue-Maclendon*
HCM pg	70		-		10	*Fortes*
Leucócitos	8693		3000 - 18500		10	*Fortes*
$/mm^3$	3600		-		-	*Green & Blue-Maclendon*
	8910		4220 - 17700		22	*Uhart et al.*
	%	**$/mm^3$**	**%**	**$/mm^3$**		
Heterófilos	62,1	5706	52 - 78	1689 - 14430	10	*Fortes*
	8,1	-	-		-	*Green & Blue-Maclendon*
	-	5805	-	2590 - 11930	22	*Uhart et al.*
Eosinófilos	2,2	176,4	0,3 - 3,1	39 - 360	10	*Fortes*
	0,2	-	-	-	-	*Green & Blue-Maclendon*
	-	690	-	84 - 1870	22	*Uhart et al.*
Basófilos	2,7	208,2	1 - 6	58 - 363	10	*Fortes*
	0,5	-	-	-	-	*Green & Blue-Maclendon*
	-	0	-	-	22	*Uhart et al.*
Monócitos	6	450,2	1,4 - 9,7	171 - 1115	10	*Fortes*
	4,3	-	-	-	-	*Green & Blue-Maclendon*
	-	635	-	176 - 2213	22	*Uhart et al.*
Linfócitos	27	2142	17,7 - 34,3	921 - 3515	10	*Fortes*
	4,3	-	-	-	-	*Green & Blue-Maclendon*
	-	1680	-	483 - 4560	22	*Uhart et al.*

Valores bioquímicos *Rhea americana*

Parâmetro	Valor médio	Rango	N	Autor
Ácido úrico (μmol/L)	317,67	172,49 - 541,27	22	*Uhart et al.*
Albumina (g/L)	25,8	14-31	22	*Uhart et al.*
Amilase (UI/L)	70,8	12-186	22	*Uhart et al.*
AST (UI/L)	43,7	16-135	22	*Uhart et al.*
Cálcio (mmol/L)	3,85	2,12-5,75	22	*Uhart et al.*
CK (UI/L)	22,3	10-63	22	*Uhart et al.*
Colesterol (mmol/L)	1,94	1,16-3,05	22	*Uhart et al.*
Fósforo inorgânico (mmol/L)	1,99	1,45-2,78	22	*Uhart et al.*
Glicose (mmol/L)	11,8	9,88-14,48	22	*Uhart et al.*
Globulinas (g/L)	22,5	15-26	22	*Uhart et al.*
K^+ (mmol/L)	4,52	3,4-5,3	22	*Uhart et al.*
LDH (UI/L)	240,7	118-445	22	*Uhart et al.*
Na^+ (mmol/L)	161,7	142-170	22	*Uhart et al.*
Proteína total (g/dL)	4,82	2,9-5,4	22	*Uhart et al.*
Razão albumina/globulinas	1,15	0,8-1,5	22	*Uhart et al.*

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