UNIVERSIDADE DE BRASÍLIA
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA

Influência do habitat na reprodução de Tiziu (*Volatinia jacarina*) no Brasil central

CARLOS HUMBERTO BIAGOLINI JUNIOR

Orientadora: Regina Helena Ferraz Macedo

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Tese apresentada ao Programa de Pós-Graduação em Ecologia da Universidade de Brasília, como parte dos requisitos para a obtenção do grau de Doutor em Ecologia

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SUMÁRIO

RESUMO

Entender o papel do habitat na reprodução dos animais é fundamental para estabelecer padrões globais de seleção sexual. Embora uma série de autores tenham explorado relações entre o habitat e a reprodução de aves, existem lacunas a serem investigadas. Este estudo tem como objetivo explorar a influência do habitat sobre a reprodução de passeriformes, usando o Tiziu *Volatinia jacarina* (Linnaeus, 1766) como modelo. Para este propósito: i) avaliamos os efeitos da oferta de alimento, intensidade de sombreamento e densidade de vegetação sobre o componente motor do display do Tiziu; ii) estimamos o impacto de infeções de berne *Philornis* sp. sobre a sobrevivência de ninhegos, e avaliamos como o habitat pode influenciar essa interação; iii) testamos se o aumento da percepção de risco de predação provoca variações em comportamentos de “trapaça” reprodutiva (i.e., fertilizações extra par (FEP) e parasitismo intraespecífico (PI)), tamanho de ninhada, razão sexual e índice de massa corporal; e iv) testamos se a complexidade da vegetação, em termos de sombra e agregação, influencia a ocorrência de FEP e PI. Detectamos evidencias da influência do habitat em diversas caraterísticas da reprodução de Tizius. A duração do salto no display é positivamente associada à oferta de alimento e intensidade de sombreamento. O aumento na oferta de alimento implica em menores taxas de infecção por *Philornis*. A percepção de risco de predação influenciou a razão sexual de ninhadas. O sombreamento do habitat aumentou a ocorrência de PI. Estas descobertas revelam novas perspectivas quanto ao papel do habitat na reprodução de aves neotropicais. Informações descritivas sobre a reprodução do Tiziu também são providas nos capítulos que compõem esta tese.. Nesse sentido, o presente trabalho amplia o conhecimento sobre a biologia reprodutiva de aves tropicais, e adicionalmente, fornece dados que permitem aprofundar a discussão acerca do tema.

Palavras-chave: corte, Thraupidae, trapaça reprodutiva, monogamia social, parasitismo, relações extra par.

ABSTRACT

Understanding the hole of habitat in animal breeding is an essential step that will contribute towards the description of global patterns of sexual selection. Although a number of authors have explored relationships between the habitat and bird reproduction, there are gaps that remain to be investigated. This study aims to explore the influence of habitat on the breeding of passerine species, using the blue-black grassquit *Volatinia jacarina* (Linnaeus, 1766) as a study model. For this purpose we: i) assessed the effect of grass seed abundance, shadow intensity and vegetation density upon the motor component of the grassquit display; ii) estimated the impact of botfly *Philornis* sp. infection on nestling survival, and evaluated how habitat can influence this interaction; iii) tested whether predation risk can induce variation in cheating behaviors (i.e., extra pair paternity (EPP) and intraspecific brood parasitism (IBP)), brood size, offspring sex ratio and body mass index; and iv) tested if habitat complexity, in terms of shadow and vegetation aggregation, influences the occurrence of EPP and IBP. We detected evidence of the influence of the habitat on several grassquit breeding traits. Display leap duration was positively associated with seed abundance and shadow intensity. Increased food supply was associated with lower rates of *Philornis* infection and predation risk perception influenced brood sex ratio. Finally, we found that habitat shadow increased the occurrence of IBP. These findings bring to the light new perspectives concerning the role of the habitat in neotropical bird reproduction. Descriptive information about grassquit breeding behavior is also provided in the chapters that compose this thesis. In this sense, the present study expands information about the breeding biology of tropical birds, providing data that allow a deeper discussion about this topic.

Key words: cheating; courtship; cuckoldry; egg dumping; fidelity; infidelity; monogamy; Thraupidae

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INTRODUÇÃO GERAL

Referencial teórico

A seleção sexual é uma poderosa força evolutiva que pode ser resumidamente definida como a escolha de caracteres que aumentam o sucesso reprodutivo de um organismo. Esta seleção atua favorecendo os indivíduos (geralmente machos) com maior capacidade de competir com outros do mesmo sexo pela obtenção de fertilizações (seleção intrasexual) ou favorecendo indivíduos que têm maior capacidade de atrair o sexo oposto (seleção intersexual) (Darwin 1871). A seleção sexual explica a evolução de caracteres ligados à competição entre machos assim como caracteres ornamentais, e a existência de conflitos evolutivos entre os sexos (Trivers 1972; Emlen & Oring 1977). De forma geral, machos competem por acasalamentos ao passo que as fêmeas selecionam cuidadosamente seus parceiros entre os competidores. Isso porque o investimento parental das fêmeas é normalmente superior ao dos machos em termos de tempo, energia e risco de mortalidade com os cuidados da prole (Trivers 1972). Enquanto os machos produzem uma grande quantidade de espermatozoides a baixo custo, fêmeas são limitadas por gerarem óvulos muito maiores (*i.e.* anisogamia), os quais frequentemente necessitam de investimento pós-fecundação (Birkhead 2010).

Entender como o habitat influencia a seleção sexual é um ponto central para o estabelecimento de padrões globais de reprodução dos organismos. Em geral, estudos que avaliam o efeito do habitat na reprodução consideram apenas elementos relacionados à obtenção de recursos, tais como: alimento (Johnson & Sherry 2001), material para construção de ninhos (Jones & Hungerford 1972; Mills et al. 1991), sitio de nidificação e refúgio (Darolová et al. 2014). Contudo, características como a capacidade de deslocamento e comunicação, que não são consumidos ou manipulados pelos organismos, também sofrem influência da estrutura física do habitat.

As aves compõem um dos táxons mais utilizados em estudos relacionados ao efeito do habitat sobre reprodução (Shannon et al. 2016). Isso é decorrente de uma série de características que tornam este táxon um excelente modelo para o tema. Primeiramente, aves são um grupo de interesse por apresentar uma enorme diversidade de padrões reprodutivos em termos de duração de temporada reprodutiva, tamanho de ninhada, tamanho de ovos, período de incubação, taxa de crescimento dos ninhegos, cuidado parental, morfologia de ovos e ninhos, entre outras características (Biddle et al. 2016). A reprodução das aves pode ser influenciada por fatores externos tais como temperatura (Biddle et al. 2018; Ospina et al. 2018), arranjo espacial de fragmentos de vegetação (Bain et al. 2014), paisagem sonora (Longcore & Rich 2004; Fuller et al. 2007; Swaddle & Page 2007; Francis et al. 2009), iluminação (Longcore & Rich 2004; Da Silva et al. 2014), fotoperíodo (Reparaz et al. 2014), fogo (Lindenmayer et al. 2014; Lantz & Karubian 2017), e eventos climáticos (Johnsen & Lifjeld 2003; Kiere & Drummond 2016). Adicionalmente, o táxon das aves apresenta uma taxonomia relativamente bem resolvida (Jetz et al. 2012) e ocupa uma enorme extensão geográfica (Myers et al. 2000; Xiao et al. 2016). Em conjunto, esses fatores permitem o estabelecimento de padrões globais que podem ser associados a variações do habitat (Biagolini-Jr et al. 2017; Menezes & Santos 2020). Nas últimas décadas, a revelação da enorme diversidade de sistemas de acasalamento provocou um aumento no interesse em estudos com o grupo (Black 1996; Ligon 1999). Testes de paternidade, baseados em DNA, revelaram que ainda que a grande maioria das aves se reproduza de forma socialmente monogâmica (Lack 1968), comportamentos de “trapaça” reprodutiva, tais como fertilizações extra par (FEP) e parasitismo intraespecífico (PI), são comuns em diversas ordens de aves (Yom-Tov 1980; Griffith et al. 2002; Biagolini-Jr et al. 2017; Brouwer & Griffith 2019).

A região tropical apresenta a maior concentração de riqueza de aves no mundo (Myers et al. 2000), contudo existe na literatura uma forte tendência a estudos realizados em áreas de regiões temperadas (Macedo et al. 2008; Xiao et al. 2016), o que dificulta o estabelecimento de padrões globais biológicos. Por exemplo, em uma revisão envolvendo dados de FEP, Biagolini-Jr et al. (2017) demonstram que apenas 15% dos trabalhos com o tema de seleção sexual tiveram dados coletados em áreas da zona tropical. Ambientes tropicais diferem de áreas temperadas, em várias características, tais como abundância e distribuição temporal de alimento, estrutura da vegetação, e condição climática (Bailey 2009; Chapin et al. 2011). Portanto, a fim de esclarecer padrões gerais da reprodução de aves, faz-se necessário o desenvolvimento de pesquisas em áreas tropicais.

Um elemento marcante de divergência entre diferentes tipos de habitat é a estrutura da vegetação, que pode ser definida como o componente físico da vegetação (Randlkofer et al. 2010). A arquitetura das plantas cria barreiras que dificultam a identificação visual (Whittingham

& Dunn 2016), auditiva (Langmore 1998; Hansen et al. 2005) e olfativa (Whittaker et al. 2013) entre indivíduos. A estrutura da vegetação também produz sombreamento, que implica em variações em luminosidade dentro de um mesmo ambiente. Para as aves, o deslocamento relativamente mais seguro poderia ocorrer em áreas de menor luminosidade. Por outro lado, o sombreamento dificulta o comportamento de vigilância de parceiro, o que pode levar ao aumento de FEP (Mee et al. 2004; Muck et al. 2009; Biagolini-Jr et al. 2017).

A disponibilidade de alimento, em termos de abundância e de sazonalidade, também é um elemento marcante na comparação entre tipos de habitat. A disponibilidade de alimento influencia uma série de fatores relacionados à reprodução, tais como: tamanho de ninhadas (Dias & Macedo 2011), morfologia dos filhotes (Hegyi & Török 2007), cuidado parental (Martínez-Padilla & Fargallo 2007), e display sexual (Manica et al. 2014). A oferta de alimento é um fator limitante para a execução de comportamentos de corte e exibições para atração de parceiros e defesa de território (Manica et al. 2014). A disponibilidade de alimento influencia a taxa de relações extra par. Em espécies com demanda elevada de cuidado parental, fêmeas são limitadas na busca por FEP frente à vigilância dos parceiros, pois quando estes detectam evidências de FEP, podem desertar o ninho (Matysiokova & Remes 2013). Dessa forma, ambientes ricos em alimento permitem que fêmeas consigam prover alimento independente da participação do macho, favorecendo a poligamia (*e.g*. Hoi-Leitner et al. 1999; Humbird & Neudorf 2008; Kaiser et al. 2017).

A predação de ninhos é reconhecidamente um fator determinante no sucesso ou fracasso reprodutivo de aves tropicais (Skutch 1985; Tori et al. 2008; Delhey et al. 2010), influenciando a evolução da história de vida das aves (Lima 2009) e moldando parâmetros reprodutivos, tais como: escolha de sítios de nidificação (Eggers et al. 2006), tamanho de ninhada (Martin et al. 2000; Eggers et al. 2006), taxa de entrega de alimento aos ninhegos (Ghalambor & Martin 2000) e período de incubação (Massaro et al. 2008). Diversos estudos indicam que devido à maior taxa de predação nos trópicos, aves tropicais apresentam menor sucesso reprodutivo do que espécies filogeneticamente próximas que ocupam áreas ne região temperada (*eg.* Francisco 2006; de Oliveira et al. 2010; Marini et al. 2014; Zima & Francisco 2016). Apesar da predação de ninhegos por vertebrados, tais como mamíferos, aves e repteis, ser frequentemente associada à diminuição do sucesso reprodutivo das aves, é crescente o reconhecimento de que a "predação" por ectoparasitas de ninhegos também podem afetar significativamente a sobrevivência da prole. Na região neotropical, infecções por larvas de moscas do gênero *Philornis,* tem sido apontadas como a principal ameaça para a extinção de algumas espécies de aves (McNew & Clayton 2018; Bulgarella et al. 2019).

Frente ao risco de predação enfrentado pelos adultos, é esperado que machos e fêmeas que busquem mais FEPs sejam favorecidos, pois seus filhotes são distribuídos em vários ninhos, aumentando a chance de que ao menos parte da prole sobreviva (Hamilton & Orians 1965; Yom-Tov 1980; Brennan 2012; Shaw & Hauber 2012). Nesse sentido, também seria possível que ao acessar um território vizinho para efetuar o parasitismo de ninho, fêmeas tivessem que copular com o macho territorial, o que também levaria a um aumento da FEP (Griffith et al. 2004). Por outro lado, se o sucesso em conquistar uma parceira estiver relacionado à exibição de display sexual (Manica et al. 2016b) e plumagem ornamentada (Doucet 2002), a obtenção de FEP poderia ser contrabalanceada por um incremento no risco de predação dos machos.

Ainda que as ideias aqui apresentadas já tenham sido discutidas por diversos autores, existem poucos estudos que sustentam a ideia de que a predação pode explicar taxas de FEP. No Chapim-real *Parus major*, as taxas de FEP estão associadas a taxas diárias de predação, qualidade dos indivíduos e densidade reprodutiva (Yuta & Koizumi 2016). Em dois estudos, a introdução de playbacks de predadores não alterou as taxas de FEP (Chapim-real: Abbey-Lee et al. 2018; Chapim-azul *Cyanistes caeruleu* (Santema et al. 2019a). Por outro lado, a introdução de modelos de predador levou a mesma população de Chapim-azul a apresentar maior incidência de FEP (Santema et al. 2019b). Já as evidências de que a predação explica em aumento no parasitismo de ninhos podem ser encontradas tanto em estudos de aves em cativeiro (Shaw & Hauber 2012) como em vida livre (Pöysä & Paasivaara 2016).

Destaco ainda que embora haja um grande volume de trabalhos publicados relacionados ao efeito do habitat na reprodução de aves, existem lacunas a serem investigadas, especialmente na região tropical. O presente estudo buscou avaliar o efeito do habitat na reprodução de espécies de aves tropicais, usando o Tiziu *Volatinia jacarina* (Linnaeus, 1766) como espécie modelo. Foi investigada a relação entre parâmetros associados ao sucesso reprodutivo e caraterísticas do habitat, tais como densidade de vegetação, sombreamento, distribuição de recursos, e risco de predação ou infecção por ectoparasitas.

Objetivos

Objetivo geral

O objetivo deste estudo é verificar a influência do habitat sobre a reprodução de uma espécie de passeriforme, o Tiziu, em uma área da região tropical.

Objetivos específicos

I. Investigar como a vegetação influência o componente motor do display do Tiziu;
II. Avaliar como o custo da infecção por larvas de mosca *Philornis* sp. afeta o sucesso reprodutivo do Tiziu, bem como avaliar como o habitat influencia esta relação;
III. Testar se o aumento da exposição ao risco de predação provoca variações em parâmetros reprodutivos do Tiziu;
IV. Investigar se a complexidade da vegetação ao redor dos ninhos influencia a frequência de FEP e IBP.

Materiais e métodos

Área e espécie de estudo

Para a realização deste estudo, foram utilizadas duas áreas de cerrado *sensu stricto*, que apresentam alta diversidade de árvores lenhosas (Assunção & Felfili 2004) e gramíneas (Aguilar et al. 2008). As áreas se encontram no Campus Universitário Darcy Ribeiro da Universidade de Brasília (UnB), DF, Brasil (15°45'S; 47°52'W; altitude média de 1020m: mínimo=959, máximo=1081), e juntas compreendem uma área total de 36 ha (Figura 1). O clima da região é classificado como Aw, pela definição de Köppen-Geiger (Kottek et al. 2006; Peel et al. 2007). A temperatura média diária é de 21,4°C (mínima 12,1°C, máxima: 29,1°C) e a pluviosidade anual média é de 1460 mm (mínima 1157 mm, máxima: 1801 mm) (INMET 2019).

Figura 1. Em vermelho: áreas utilizadas para a realização do presente estudo inseridas no campus da Universidade de Brasília (15°45'S; 47°52'W), com uma área total de 36 ha.

O Tiziu é uma espécie abundante no Brasil central, apresentando um sistema de acasalamento caracterizado pela monogamia social, com ocorrência de FEP, considerado um tipo de “trapaça” reprodutiva (Carvalho et al. 2006; Manica et al. 2016a). Durante os meses de novembro a abril, ocorre um aumento da população do Tiziu no Brasil central, associado ao aumento da pluviosidade e da disponibilidade de sementes de gramíneas (Dias et al. 2009). Durante esse período os pares de tizius constroem ninhos em arbustos, touceiras de capim ou bambus (Poaceae: bambusoideae), dentro de pequenos territórios de 13 a 72 m^2 (Almeida & Macedo 2001), defendidos por machos que realizam exibições de corte (“display” sexual) ao longo de todo o dia durante toda temporada reprodutiva (Carvalho et al. 2007) .

A escolha de local para nidificação é relacionada à estrutura da vegetação (Aguilar et al. 2008). Ninhos são espacialmente agregados, o que leva a um aumento local da predação (Dias et al. 2009), fator este considerado o principal responsável por fracassos na reprodução da espécie (Almeida & Macedo 2001; Carvalho et al. 2007; Aguilar et al. 2008; Dias et al. 2010). A suplementação de alimentos provoca modificações na frequência de disputas territoriais, frequência de visitações por fêmeas, número de filhotes gerados em cada ninhada, taxa de crescimento dos filhotes, bem como modificações na taxa de predação (Dias & Macedo 2011).

Delineamento experimental e coleta de dados

A reprodução de Tizius foi monitorada ao longo de quatro temporadas reprodutivas (2015-2019). Durante as duas primeiras temporadas reprodutivas (2015-2016; 2016-2017), foi realizado um experimento para testar o efeito da percepção do risco de predação na reprodução do Tiziu (Capítulo 3). Nas demais temporadas (2017-2018; 2018-2019) a reprodução do Tiziu foi monitorada sem realização de manipulação experimental, e dados ecológicos de interesse foram coletados a fim de testar o efeito do habitat sobre o componente motor dos displays (Capítulo 1), o efeito de ectoparasitas na reprodução do Tiziu (Capitulo 2) e o efeito da vegetação sobre as taxas de FEP (Capítulo 4).

A identificação de territórios e a busca por ninhos foram realizadas por meio de caminhadas lentas nas áreas de estudo, utilizando sempre que necessário uma haste para examinar moitas e arbustos, e binóculos (10x42 Zeiss-Terra ED) para acompanhar o movimento de adultos. Poleiros utilizados para displays (capítulo 1) e ninhos (capítulos 2, 3 e 4) foram georreferenciados por meio de GPS de alta precisão (modelo GPSmap 62st Garmin). Ninhos vazios foram marcados e revisitados no período de 1-2 semanas para avaliar a ocorrência de atividade. Os métodos para amostragem de material biológico dos ninhegos variaram entre os estudos, e informações detalhadas são apresentadas no item métodos de cada capítulo.

A fim de identificar parentais sociais e realizar testes de paternidade, foi realizada a captura de adultos com redes de neblina (Roos 2010), semanalmente, ao longo de todas as temporadas reprodutivas, no período da manhã (das 05:00 as 12:00 h). Eram sempre dispostas de 8 à 12 redes de nylon (2 x 12 m) em pontos aleatórios das áreas de estudo. As redes eram checadas em intervalos de 20-30 minutos, e as aves capturadas mantidas em sacos de pano de tecido poroso. Os indivíduos capturados foram marcados com uma combinação única de anilhas coloridas de PVC e uma anilha metálica numerada, para futura identificação. Dado a possibilidade de influência da coloração das anilhas na seleção sexual (Zann 1994; Johnsen et al. 1997; Johnsen et al. 2000), buscamos minimizar viés na escolha de combinações de cores de anilhas, utilizando o algoritmo *Variable frequency*, descrito em Biagolini-Jr & Macedo (2019), implementado no pacote GenTag (Biagolini-Jr 2019), desenvolvido para o software R. As aves capturadas foram pesadas com dinamômetro com capacidade de 20g e precisão de 0,2g (Pesola

Light line 10020), e também foram tomadas medidas morfométricas dos tarsos, asas, cauda e bico. Amostras de sangue (aproximadamente 50 µL) foram coletadas de todos animais anilhados com capilar por meio de punção da veia braquial (Owen 2011), e armazenadas em álcool etílico absoluto PA (J.T. Baker 9014-02).

Destacamos que a identificação da paternidade social por identificação de qual macho realiza display mais próximo ao ninho é de baixa confiabilidade, pois os ninhos tendem a ser construídos uns próximos aos outros (Aguilar et al. 2008). Por esse motivo, buscamos a identificação dos parentais sociais por meio de filmagem dos ninhos, mas mesmo utilizando tal metodologia, apenas 6% dos ninhos (23 de 361) tiveram ambos parentais identificados com anilhas. Dado o grande volume de ninhos sem a identificação dos parentais sociais, optamos por utilizar ferramentas moleculares para identificar o grau de parentesco entre os ninhegos, e estimar a incidência de fertilização extra par de forma indireta. Tal procedimento é comumente aplicado quando não é possível amostrar ou confirmar a paternidade social (*eg.* Mino et al. 2011; Lopes et al. 2013; Turjeman et al. 2016). Nesse contexto, casos onde foram detectados filhotes de um mesmo ninho que compartilhavam apenas um parental, *ie.* meio irmãos, foi assumido que houve FEP. Já aqueles filhotes em um mesmo ninho que não compartilhavam nenhum parental foram considerados como resultantes de PI.

Ao longo de quatro temporadas reprodutivas foi realizado um esforço aproximado de 53276 m^2/horas, foram anilhados 1519 adultos de vida livre, sendo 771 machos e 748 fêmeas ou jovens com sexo indefinido. Somando o esforço de campo no uso de redes de neblina, busca e monitoramento de ninhos, e coleta de dados ambientais, o presente estudo teve um esforço de aproximadamente 1952 horas de campo.

Bioética e licenças para execução do projeto

O presente estudo foi autorizado pelo Sistema de Autorização e Informação em Biodiversidade (SISBIO) (licenças n^{os}. 51639 e 60652). O anilhamento foi autorizado pelo Centro Nacional de Pesquisas para Conservação das Aves Silvestres (CEMAVE) (licenças n^{os}. 4255 e 4266). O acesso ao patrimônio genético, foi registrado no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGen) sob cadastro de acesso de nº A3D8AEB. O projeto também foi aprovado pela Comissão de Ética no Uso Animal (CEUA) da Universidade de Brasília (Doc nº 66711/2016).

Formatação da tese

Todos os capítulos desta tese encontram-se formatados como artigos científicos, sendo que o primeiro capítulo já foi submetido ao periódico *Journal of Avian Biology*. Os demais capítulos estão formatados, respectivamente, de acordo com as normas das revistas científicas *Journal of Field Ornithology*, *Journal of Animal Ecology* e *Behavioral Ecology and Sociobiology*, e serão submetidos após a apreciação da tese pela banca examinadora.

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CAPÍTULO 1

Food and shadow influence a tropical bird display

Este manuscrito foi submetido ao periódico *Journal of Avian Biology*. Portanto, este segue o padrão de referência bibliográfica do periódico

Abstract

Resource distribution patterns are directly associated with territorial disputes among individuals. Frequently, the type of vegetation coverage and its complexity reflect the abundance of resources and also provide the backdrop for most animal activities. The degree of vegetation complexity can either facilitate or hinder daily activities of individuals, since high vegetation density reduces the probability of detection by predators. For instance, conspicuous displays may be safer when conducted in territories with a high degree of vegetation complexity. The aim of this study was to evaluate the relation between motor display parameters of the blue-black grassquit (*Volatinia jacarina*) and vegetation complexity. We expected to find that males in more complex vegetations would perform more conspicuous courtship displays. We modelled two display parameters that render the individual more conspicuous and also reflect higher energetic motor investment (leap duration and frequency) and their relation with three habitat characteristics that are indicative of vegetation complexity (grass seed abundance, shadow intensity and vegetation density). We found that leap duration increases with high seed abundance and shadow intensity. We also found evidence that food abundance is the main predictor of leap duration. Display frequency, *ie.* number of leaps per minute, was not associated with any vegetational parameter. Our results show that shadow intensity plays a strong role in shaping some aspects of animal behavior, highlighting the fact that physical barriers can impact sexual selection. Our findings also extend awareness about the interpretation of courtship exhibitions as honest signals of territory quality.

Key words: Agonistic behavior; Breeding; Courtship; Ornament; Sexual selection

Introduction

The vegetation that composes different habitats is usually considered as a source of food (Johnson and Sherry 2001), building material for nests or refuges (Darolová et al. 2014), or as a substrate for nest sites (Jones and Hungerford 1972, Mills et al. 1991). However, only a fraction of a vegetation´s diversity is used for these purposes. One aspect of vegetation that is seldom considered when evaluating its context and impact upon an animal´s behavior is its structural complexity, which can be defined as plant architecture or connectivity of plant parts, which reflects the underlying diversity of plant species (Randlkofer et al. 2010).

Vegetation complexity is linked to at least two important attributes that could influence animal behavior. The first one is the level of concealment that the vegetation provides to its animal inhabitants due to physical barriers that limit vision, sound propagation and movement (Norris and Stutchbury 2002). Such physical barriers contribute positively towards prey survival in the environment, as they reduce detectability. However, physical barriers can also restrict conspecific detection based on vision (Whittingham and Dunn 2016), hearing (Langmore 1998, Hansen et al. 2005) and odor (Whittaker et al. 2013). Likewise, vegetation structural complexity also leads to variations in shadowing, which influences animal detectability (Galeotti et al. 2003, Wilson and Watts 2006). Under low luminosity, individuals are less likely to be detected by predators (Götmark and Hohlfält 1995, Fernández-Juricic et al. 2012) or conspecifics (Sicsú et al. 2013, Ward et al. 2014). Thus, daily displacement should be safer in shadowed parts of the habitat or under low luminosity conditions, which occur at dawn or dusk (Double and Cockburn 2000, Schlicht et al. 2015). The second attribute that results from vegetation complexity is its productivity in terms of food availability in the form of leafy materials, seeds, buds and flowers. These associations suggest an interdependence between bird dispersal patterns of seeds and vegetation structure and composition (Yarranton and Morrison 1974).

Vegetation complexity, by influencing both concealment and food availability in a bird´s natural habitat, may strongly influence specific behavioral components involved with breeding. High vegetation density, for example, could provide higher cover for conspicuous or singing males during courtship behavior. Vegetation density may also be an obstacle for mate-guarding, making it more difficult for individuals to monopolize sexual partners (Mays and Ritchison 2004). There is strong evidence that birds attempt to conceal themselves during extra-territorial forays, and in females this occurs more frequently during her fertile periods (Humbird and Neudorf 2008), possibly when searching for extrapair mating opportunities. Female concealment during extra-territorial forays may be a strategy to avoid the consequences of male behavior in face of cuckoldry, such as decreased paternal investment (Suter et al. 2009, van Dijk et al. 2010). Thus, in theory, increased vegetation complexity should promote polygamy (Mee et al. 2004, Muck et al. 2009). Additionally, higher food abundance in areas with more complex vegetation could also promote more vigorous competitive behaviors among males.

In this study we asked whether vegetation complexity can influence sexual selection using the Neotropical blue-black grassquit *Volatinia jacarina* as our model animal. The blue-black grassquit is a granivorous passerine that breeds in savanna regions and altered habitats of varying complexity (Almeida and Macedo 2001). Throughout the breeding season males engage in multimodal sexual displays that include visual and vocal components. The blue-black grassquit is an excellent study species for our objectives because of its dependence upon vegetation complexity in various contexts. For instance, under direct sunlight males increase their displays rates (Sicsú et al. 2013), suggesting that sunlight patches and their distribution in the habitat are of importance. Males need elevated perches to do their leaping displays, as this possibly augments the reach of their vocal output (Wilczynski et al. 1989). Furthermore, the shadows produced by the vegetation around the display perches create blind spots for hiding within the vegetation. Because predation of grassquit eggs and nestlings is very high (Macedo et al. 2012), the existence of hiding places could be extremely important to deter predators. Previous studies of grassquits have indicated that predators use displaying males as cues for finding nests (Dias et al. 2010) and high-quality males avoid displaying in the presence of a predator decoy (de Moraes et al. 2019). Vocal output during displays honestly indicates territory quality in the form of seed abundance (Manica et al. 2014), and females prefer to pair socially with males that perform higher leaps(Manica et al. 2016a). Thus, for all these reasons, vegetation complexity may be a parameter of extreme importance for the survival and breeding success of the blue-black grassquit

We hypothesized that blue-black grassquit males in more complex vegetations would perform more energetic courtship displays. We based this hypothesis on two assumptions. We assume that in more complex vegetation landscapes males should be more active because the vegetation confers a higher degree of concealment, which can be interpreted in two ways. Complexity in vegetation may lower predation risk for both adults and offspring within the lower strata of the vegetation (Zuk and Kolluru 1998, Stuart-Fox et al. 2003), and could also provide the possibility for males to increase their courtship investment at a lower predation risk. Alternatively, vegetation complexity may be an obstacle for a male to gain high visibility during display activities. Thus, the complexity of the vegetation may lead to higher display activities because, despite remaining better concealed in their regular activities, males have to increase their energetic investment in displaying to gain visibility in courtship contexts. A second assumption of our hypothesis is that more complex vegetations yield higher food availability, which could provide energetic resources to drive more intense and vigorous motor displays.

Methods

Study site and species

We carried out this study from December to March across two breeding seasons (2017-2018 and 2018-2019) within the University of Brasília campus, in Brasília, Brazil (15°45'S; 47°52'W; altitude *ca* 1000 m). The vegetation in the study site is classified as Cerrado *sensu stricto* (tropical savanna) with a high diversity of woody trees (Assunção and Felfili 2004) and the occurrence of invasive grasses such as *Brachiaria* sp. and molasses grass *Melinis minutiflora*. The average daily temperature was 21.4° C (minimum 12.1 ° C, maximum: 29.1 ° C), average annual rainfall was 1459.6 mm (minimum: 1157.1 mm, maximum: 1801.3 mm) (INMET 2019) and the Köppen-Geiger climate classification is Aw (Kottek et al. 2006, Peel et al. 2007).

The blue-black grassquit is a species commonly found in grasslands throughout the Neotropical region. In central Brazil, where this study was conducted, there is an increase in its population size between November to April, which is associated with the rainy season. During this period males molt to a blue-black iridescent nuptial plumage. During the non-breeding season males and females have a light brown plumage coverage (Marcondes-Machado 1987; Moreno-Palacios et al. 2013).

During the breeding season, blue-black grassquits perform multimodal displays through most of the day (Carvalho et al. 2007) from within the limits of their small territories (13.0 to 72.5 m^2; Almeida and Macedo 2001). The male display comprises a short vertical leap (average height of approximately 20 cm; Manica et al. 2016b) initiated from a perch, accomplished through a variable number of wingbeats, and synchronized with a strident vocalization, resulting in a visual and acoustic stimulus for conspecifics (Manica et al. 2014, Manica et al. 2016a). Displays are assumed to be energetically costly (Manica et al. 2016b), and can be performed multiple times per minute (see results).

Nest site selection is based on microhabitat complexity (Aguilar et al. 2008), and nests are spatially aggregated, which leads to a local increase in population density and predation (Dias et al. 2009). Both sexes build the nest and provide parental care (Carvalho et al. 2007), but extrapair paternity is exceptionally high (Carvalho et al. 2006, Manica et al. 2016a). Predation is considered the main factor leading to lack of breeding success (Almeida and Macedo 2001, Carvalho et al. 2007, Dias et al. 2010).

Data collection

Territories were identified by taking slow walks within the study area to visualize displaying males. In morning hours (0630-1100), period of high activity for birds, we video recorded male displays with digital cameras (Canon EOS Rebel T5 or Sony CX405, at 60 fps) attached to tripods at distances of 10-20 m. Previous studies indicate that leap parameters (*eg.* height, duration, rotation angle, launch velocity, and number of wing beats) are strongly correlated (Manica et al. 2016b), and mathematical modeling using different parameters converges to the same result (*eg.* de Moraes et al. 2019). Leap duration is preferred over other parameters because it is easily recorded using digital videos; and does not require the use of image analysis software (such as rotation angle and launch velocity). Given the nature of the leap duration variable (continuous), there is a further advantage that the mathematical modelling does not require adjustments to the distribution of the response variable, making mathematical modeling simpler and easier to interpret. Therefore, we used only two independent display parameters indicative of the bird's vigor: leap duration and leap frequency. We used a video player software (MPC-HC Version 1.7.9.181) to extract leap duration (in milliseconds). Leap frequency was calculated as the number of leaps divided by minutes of display performance. We assumed that the leap itself started on the first video frame where the bird´s claws were no longer in touch with the perch and ended on the last video frame before the claws touched the perch again. Video recording started as soon as the camera was set up, but we recorded display bouts only when the bird executed its first leap, to avoid differences due to individual boldness. The display bout was usually interrupted due to territorial disputes, or less frequently due to foraging, for pair-bond interactions or nestling attendance. In such cases, we assume that the display bout comprised the period of time between the beginning of the first leap and the beginning of the last leap recorded in sequence. We excluded display bouts with fewer than 10 leaps.

Within seven days after video recording took place, we assessed vegetation complexity by averaging habitat parameters sampled at five spots at distances of 3 m from the display perch used by each focal male. These non-correlated parameters (see Results) included shadow intensity, seed abundance and vegetation density. To avoid variations in shadow intensity given sun position, we collected data from habitat parameters in morning hours (0630-1100), in the same time period that displays were recorded. To avoid changes in shadow patterns due to movements in the vegetation while collecting the data, we first collected the data to estimate shadow strength, followed by abundance of seed resources and vegetation density.

Shadow intensityrepresents a static measure of the proportion of sunlight captured by the vegetation. In the field, the amount of sunlight that arrives at the top of the vegetation in all territories tends to be the same, however the intensity with which light is absorbedby the vegetation varies among territories. As natural changes in vegetation through growth are slow, the shadow strength within each territory can be considered constant across the breeding season. We estimated shadow strength by calculating the slope of a linear regression of light-meter measures (Extech 401025) and height at which each measure was taken: 200, 180, 150, 120, 50, 40, 30, 20 and 10 cm above ground. Despite the natural variation of maximum vegetation height, most of the vegetation in the study was below 200 cm. All light measures were taken in approximately 20-30 seconds, values were voice recorded in smartphones and followed up with computer transcriptions.

We estimated the abundance of seed resources in plots of 50 x 50 cm (Manica et al. 2014) by counting the number of seed inflorescences in each plot and then multiplying this number by the average number of seeds contained in 10 seed inflorescences collected at random. Vegetation density was estimated in plots of 30 x 100 cm by adaptation of the Zehm et al. (2003) method. A photograph was taken of the vegetation against a panel of 100 x 100 cm white cloth placed perpendicularly to the ground on the 100 cm side of the plot. The photograph was converted to a pure black and white image in GIMP software (version 2.8.22). Vegetation density was estimated as the proportion of black pixels relative to total number of pixels. Image processing was performed in the Bwimage package version 1.0 (Biagolini-Jr 2019; see Biagolini-Jr & Macedo (2019) for details).

Data analyses

To evaluate if display parameters (response variables) were associated with habitat characteristics (explanatory variables), we created multiple linear regression models for each display parameter, *ie.* leap duration and frequency. In a preliminary analysis, we tested if grass seed abundance, shadow intensity and vegetation density were correlated. Since the variables were uncorrelated (see Results), we used all three as predictors for mathematical modelling (Zuur et al. 2009).

We defined global models containing all predictor variables and their possible interactions. We then performed model selection by backward stepwise model selection, using likelihood ratio tests criteria to sequentially test which terms should be dropped to arrive at a minimal adequate model (MAM). The best model is achieved when all variables have significant effects and when the removal of any term from the model implies a significant decrease in model fit. No evidence to support the tested hypothesis is accepted when none of the predictor variables (null model) is present in MAM.

All predictor variables were scaled to mean 0 and standard deviation 1. Transforming predictor variables improves model performance and interpretability (Gelman and Hill, 2007). For MAMs with multiple predictor variables, we checked collinearity by variance inflation factor (VIF), calculated by vif function from the car package version 3.0 (Fox et al. 2019). We assumed the threshold of 3 as evidence for colinearity (Zuur et al. 2010, Fox and Weisberg 2018). We checked model validation by graphic inspection of homogeneity, residuals normality, and independence of each explanatory variable (Zuur et al. 2009). Here, we stated that models follow all criteria for model selection: there was no evidence of non-homogeneity or of dependence between residuals, and explanatory variables and residuals were normally distributed. There was no evidence of collinearity between the predictor variables.

The relative importance of each predictor variable was estimated by calculating squared partial correlations. Squared partial correlations measure the correlation between the response variable and each explanatory variable, after removal of all known effects of the remaining explanatory variables. Squared semipartial correlations yield the proportion of variance in the display parameters that are uniquely associated with each explanatory variable (Howell 2012). Partial and semipartial correlations were calculated by lmSupport package version 2.9 (Curtin 2018). All analyses were conducted in R software (version 3.5.0). All values are presented as mean ± standard deviation, unless otherwise noted, and the alpha level was set at $p = 0.05$.

Results

We successfully recorded forty males performing displays, and video records contain an average of 19.47 ± 8.27 leaps (range 11-53) per bout. Leap duration was 462.53 ± 69.55 ms (range 318.77-683.78) and leap frequency averaged 14.05 ± 2.80 leaps/min (range 7.32-19.14). The rest time between leaps was 4.00 ± 1.05 sec (range 2.62-7.79). No correlation was detected between habitat parameters, *ie.* seed resources and shadow intensity ($t = 1.19$, $df = 38$, $p =$

0.241), seed resources and vegetation density (t = -0.67, df = 38, p-value = 0.507), and shadow intensity and vegetation density (t = 0.769, df = 38, p = 0.447). The models targeting the leap duration response variable (Model I) show significant positive correlations relative to seed resources and shadow intensity. Seed resources and shadow intensity present, respectively, squared partial correlations of 18.6% and 11.2%, and squared semipartial correlations of 15.6% and 8.9% (Table 1). On the other hand, leap frequency was not related to any habitat parameters (Model II). We looked for potential outliers, and tested other model distributions, but results remained the same. Thus, we assume that there is no evidence that leap frequency could be explained by the set of explanatory variables we investigated. Backward stepwise model selection steps are presented in the Supplementary material.

Discussion

Understanding the role of habitat in shaping animal behavior is a challenge for behavioral ecologists. Here we show that habitat characteristics can influence the acrobatic displays of the blue-black grassquit, a Neotropical bird. Leap duration increases with the abundance of food resources (measured as number of grass seeds) and shadow intensity within the male’s territory. A previous study with this species showed that display vocal output was positively associated with territory quality, evaluated in terms of seed abundance (Manica et al. 2014). Because vocal output is potentiallycorrelated with leap performance variables , our expectations of finding an association between leap attributes and food resources were supported.

Our findings confirm previous suggestions that the motor component of the grassquit display is an honest signal of the quality of the male´s territory. This honest signal may reflect two possibilities. First, by holding a food-rich territory, males need less time to locate food and can spend more time on courtship motor displays or singing (Manica et al. 2014, McLeod and Ritchison 2018). However, conspicuous grassquit males attract predator attention (de Moraes et al. 2019), and possibly, only high-quality individuals can sustain exuberant traits (Saino et al. 1997, Boyd et al. 2018, Cantarero et al. 2018). This second possibility goes hand-in-hand with the Handicap Principle (Zahavi 1975).

Resource restriction is one of the tenets of sexual selection (Dillard and Westneat 2016), and maintenance of a high-quality territory is critical for male, female and offspring success. The correlation between food supply (*ie*. high-quality territory) and display vigor, explicit in our results, is probably linked to male-male competition and reflects an honest signal of male quality. From the male’s perspective, polygamy is advantageous relative to monogamy only when food is available (Herényi et al. 2014). From the female’s perspective, both quantity (Siikamäki 1995, Kaiser et al. 2015) and quality (Kankova et al. 2014, Siitari et al. 2015) of the eggs can increase with food availability and female nutritional status. From the offspring’s perspective, its nutritional condition during the nestling period may impact its adult life and success (Nowicki et al. 1998, Hegyi and Török 2007, Krause et al. 2017). In a previous study, experimental manipulation of food abundance in grassquit territories increased territorial disputes, frequency of female visits, number of eggs per clutch and nestling growth rates (Dias and Macedo 2011).

Our finding relative to the effect of shadowing on leap duration reveals the role of a rarely considered habitat parameter relative to courtship displays. This is the case not only for our study bird, the blue-black grassquit, but also for other animals where displays are critical for mate attraction but also attract predators. The increase in shadowing strength leads to more vigorous displaying. This result suggests that regardless of food abundance, displays are also influenced by the possibility of hiding in the shadows provided by the vegetation. This finding is puzzling when confronted with previous results for this same species, where it was found that individuals bathed in sunlight increased the frequency of their leap displays (Sicsú et al. 2013). However, the previous study concerned only sunlight at the top layer of the vegetation. Here, we show that the various strata of vegetation, and its complexity, are also important components that regulate intensity of displays. Previous studies with other bird species also suggest that physical barriers within territories play a role in sexual selection (Mays and Ritchison 2004).

Shadowing provides safety for birds to move inside their territories with lower detectability from both adult and nest predators. Previous studies with the blue-black grassquit showed that high quality males reduce their display rates when subjected to predation risk (de Moraes et al. 2019) and that artificial nests in the vicinity of displaying males suffer higher predation (Dias et al. 2010). Thus, in the typical grassquit habitat, a structurally simple savanna landscape, shadowed areas within a territory could be a valuable asset to decrease susceptibility to predation. Nest sites would also benefit from higher shadowing, since tropical birds face high rates and diversity of predators (Martin 1993, Stutchbury and Morton 2001, Menezes and Marini 2017).

In conclusion, our results suggest that the energetic investment into display activities in blue-black grassquits is modulated by both food availability and shadowing patterns within territories, characteristics that are indicative of structural complexity of the vegetation. Further studies should explore whether natural food supply and shadowing strength can impact other components of sexual selection in this species, such as extra pair fertilization rates.

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Conflict of interest - The authors declare that they have no conflict of interest.

Permits - This survey is part of a project to explore the role of habitat in blue-black grassquit breeding biology. Methods used in this study are in accordance with ethical standards and Brazilian laws. The relevant licenses for this project are: Bioethics Committee of Universidade de Brasília license no. 66711/2016; Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) license no. 51639; and Centro Nacional de Pesquisas para Conservação das Aves Silvestres (CEMAVE) license no. 4255.

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Table

Table 1. Model I coefficients estimation (β), standard errors (Std. Error), F and p values from term significance test, squared partial correlation (SPC) and squared semipartial correlation (SSC) index. The minimal adequate model formula is Leap Duration ~ Grass Seed Abundance + Shadow Intensity + ε.

Coefficients	β	Std. Error	F-value	p	SPC	SSC
Number of Seeds	0.406	0.14	8.415	<0.001	0.186	0.156
Shadowing	0.303	8. 0.14	4.685	0.037	0.112	0.089

Supplementary material

Table S1. Backward stepwise model selection of Model I, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model (MAM). Response variable: LeapDuration; predictor variables: GrassSeedAbundance (GSA), ShadowIntensity (SI), and VegetationDensity (VD).

Step	Term	Df	Sum of Sq	AIC	F value	p
1	<none>			-7.8654		
	GSA:SI:VD	1	0.45793	-9.0423	0.6653	0.4207
	Step 1 conclusion: drop term GSA:SI:VD					
2	<none>			-9.0423		
	GSA:SI	1	0.08533	-10.8908	0.1252	0.7257
	GSA:VD	1	0.89408	-9.4826	1.3122	0.2602
	SI:VD	1	0.58975	-10.0067	0.8656	0.3589
	Step 2 conclusion: drop term GSA:SI					
3	<none>			-10.891		
	GSA:VD	1	0.81316	-11.475	1.225	0.2762
	SI:VD	1	0.75774	-11.57	1.1415	0.2929
	Step 3 conclusion: drop term SI:VD					
4	<none>			-11.57		
	SI	1	5.5516	-5.0306	8.3295	0.00664
	GSA:VD	1	2.4377	-9.5942	3.6575	0.06403
	Step 4 conclusion: drop term GSA:VD					
5	<none>			-9.5942		
	GSA	1	5.3027	-4.1082	7.4091	0.009938
	SI	1	4.049	-5.7559	5.6573	0.02281
	VD	1	1.4306	-9.4327	1.9989	0.166002
	Step 5 conclusion: drop term VD					
6	<none>			-9.4327		
	GSA	1	6.1848	-3.2362	8.4144	0.006233
	SI	1	3.4438	-6.6635	4.6853	0.036946
	Step 6 conclusion: MAM = LeapDuration ~ GrassSeedAbundance + ShadowIntensity + ε					

Table S2. Backward stepwise model selection of Model II, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model (MAM). Response variable: Frequency; predictor variables: GrassSeedAbundance (GSA), ShadowIntensity (SI), and VegetationDensity (VD).

Step	Term	Df	Sum of Sq	AIC	F value	p
1	<none>			5.8045		
	GSA:SI:VD	1	3.2274	7.766	3.3315	0.07731
	Step 1 conclusion: drop term GSA:SI:VD					
2	<none>			7.766		
	GSA:SI	1	0.87989	6.7813	0.8483	0.3637
	GSA:VD	1	0.01876	5.7879	0.0181	0.8938
	SI:VD	1	0.61531	6.4787	0.5932	0.4466
	Step 2 conclusion: drop term GSA:VD					
3	<none>			5.7879		
	GSA:SI	1	0.86951	4.7908	0.8633	0.3594
	SI:VD	1	0.71126	4.6102	0.7061	0.4066
	Step 3 conclusion: drop term SI:VD					
4	<none>			4.6102		
	VD	1	0.88981	3.6156	0.8909	0.3517
	GSA:SI	1	0.6627	3.3614	0.6635	0.4208
	Step 4 conclusion: drop term GSA:SI					
5	<none>			3.3614		
	GSA	1	0.33159	1.732	0.3351	0.5663
	SI	1	2.32944	3.8952	2.3543	0.1337
	VD	1	0.51628	1.937	0.5218	0.4747
	Step 5 conclusion: drop term GSA					
6	<none>			1.732		
	SI	1	2.06935	1.97053	2.1297	0.1529
	VD	1	0.64576	0.44409	0.6646	0.4202
	Step 6 conclusion: drop term VD					
7	<none>			0.44409		
	SI	1	2.4024	0.98729	2.4945	0.1225
	Step 7 conclusion: MAM = Frequency ~ 1+ ε					

CAPÍTULO 2

Impact of parasitic botfly on blue-black grassquit nestlings

Este manuscrito será submetido ao periódico *Journal of Field Ornithology* após a apreciação pela banca examinadora. Portanto, este segue o padrão de referências bibliográficas do periódico.

Abstract

Parasitic botfly larvae (*Philornis* ssp., Diptera: Muscidae) are found in nests of several bird taxa, although prevalence and nestling tolerance vary considerably among species. Here we describe patterns of botfly infestation in blue-black grassquit (*Volatinia jacarina*) nestlings. We identified the most typically affected nestling body parts and assessed parasite prevalence, impact on nestling survival, changes in nestling body shape and mass index. Additionally, we test whether climatic conditions, nest morphology and habitat characteristics are associated with larvae abundance. Blue-black grassquits had low breeding success (15%), but most failures result from predation by vertebrate predators. We estimated that only 1% of nestlings died due to botfly infestation, and the number of larvae in nestling body did not change nest success. Infected chicks exhibited a higher body mass to tarsus length ratio, and higher tarsus asymmetry. Previous studies indicate that adult grassquits with a higher body mass index show lower dominance status and low mating success. Thus, we argue that although botflies had a small impact on offspring survival, they may reduce fitness in adulthood. There was no evidence that environmental conditions and nest morphology are linked to the number of larvae on nestlings. Territories with higher food supply had lower infestation rates. Possibly, food-rich habitats allow parents to spend more time on parental care (incubating nestlings), protecting them from fly attacks. The present study brings to light new perspectives concerning bird-botfly interaction.

Introduction

Botflies (*Philornis* ssp, Diptera: Muscida) are avian nest parasites that comprise about 50 species, geographically distributed from the United States to Argentina (De Carvalho et al. 2005, Dudaniec and Kleindorfer 2006, Teixeira 1999). Adults feed on decaying organic matter or flowers (Fessl et al. 2001), while larvae feed on feces (coprophagous scavengers), blood (semi-haematophagous parasites), or nestling tissue and fluids (subcutaneous parasites) (Dudaniec and Kleindorfer 2006). Pupation occurs at the bottom of the nest (Fessl et al. 2006, Saravia-Pietropaolo et al. 2018), pupation lasted on average 10.5 days and adults lived up to 100 days (Saravia-Pietropaolo et al. 2018). Infection has been reported for over 160 bird species (revised by: McNew and Clayton 2018), and occurs more often in passerines (Antoniazzi et al. 2011). Overall, *Philornis* infection risk is determined by a combination of several factors, including host taxa, environmental conditions, host life history, and habitat around the nest site.

The probability of a nest to be infected depends on host taxa, it is easily recognized when comparing infection rates of two, or more, host species living in a same area (Knutie et al. 2017, Lopes and Marini 2005, Rabuffetti and Reboreda 2007). Infection is usually linked with decreased body condition of nestlings (*eg.* O'Connor et al. 2010a, Hayes et al. 2019, Norris et al. 2010, Uhazy and Arendt 1986). Some taxa are naturally tolerant to botfly parasitism so that even when infestation occurs, there is no decrease in nestling survival (Mezquida and Marone 2001, Cockle and Bodrati 2009, Norris et al. 2010). In some cases, nestlings survive larval infection but suffer negative impacts in adulthood. This may result from nestlings fledging with a low body condition and/or due to changes in body shape that affect sexually selected traits (Pérez-Rodríguez et al. 2017). For instance, Darwin's finches infected by *P. downsi* produced songs with lower maximum frequency and greater vocal deviation, which results in indistinguishable vocalizations from different species, potentially facilitating hybridization (Kleindorfer et al. 2019). Ectoparasites can also influence other secondary sexual traits such as nestling body asymmetry (Brown and Brown 2002). Although asymmetry has a small impact on annual survival (Brown and Brown 2002), increased degree of asymmetry entails decreased fitness because it is associated with secondary sexual characters (Evans 1993, Manning and Hartley 1991).

Several studies link increased levels of rainfall and humidity to higher success of botfly larvae (Antoniazzi et al. 2011, Arendt 2000, Langen and Berg 2016, Manzoli et al. 2013, Nores 1995). As an adaptive response, it has been suggested that some birds shift their breeding season to drier months when botfly densities are low, but this implies low food availability for nestlings (Langen and Berg 2016). On the other hand, tolerance to botflies may increase in years of higher humidity when parents have sufficient resources to compensate the negative effect of parasitism (McNew et al. 2019).

Despite variations in air humidity and temperature, botflies are favored by the microclimate generated by nest architecture and building (Kleindorfer and Dudaniec 2009). Parasites are sensitive to minimal changes in nest temperature, and nestlings body temperature can improve larval development (Sage et al. 2018). Interspecific comparisons point out that species´ nest type (open versus closed) do not change infection (Dudaniec and Kleindorfer 2006, Quiroga et al. 2012), but small twigs in the nest increase larvae abundance (Quiroga et al. 2012). Twigs buried in the nest walls can create holes that favor access of adult botflies to chicks during egg laying. When the larvae move to the bottom of the nest for pupation, a denser nest wall protects them from removal attempts by the parents (Kleindorfer and Dudaniec 2016) or by predators, such as ants (Knutie et al. 2017, O'Connor et al. 2010b). Some secondary components of plant species can act as parasite repellents and may be used in nest construction (Clark 1990, Quiroga et al. 2012, Wimberger 1984). Overall, however, little is known about how intraspecific variations in nest architecture and nesting materials affect infection risk.

The area selected for building the nest could influence infection risk due to variations in vegetation patterns and population density. If areas with high vegetation density and food availability lead to high host density, an increase in infection rate is expected because the distance between neighbors nest are reduced (Antoniazzi et al. 2011, Kleindorfer and Dudaniec 2009). On the other hand, denser vegetation around the nest filters olfactory and visual cues used by parasites for nest detection. It has been shown that infection is inversely correlated to vegetation height (Manzoli et al. 2013). Furthermore, high host food availability can increase chance of botfly complete it’s life cycle, since birds can compensate for energy loss due to nest parasitism by increasing food provisioning behavior parents, *ie*. food compensation hypothesis (Hurtrez-Boussès et al. 2000, Johnson and Albrecht 1993, Tripet et al. 2002, Tripet and Richner 1997).

The aim of this study was to describe the patterns of botfly infection in a population of Neotropical blue-black grassquits *Volatinia jacarina* in central Brazil. We expected to find that infection rate was negatively correlated to offspring success, and that it also affected nestling body shape and condition. Additionally, we tested whether climatic conditions, nest architecture and habitat characteristics are associated with botfly abundance. In this regard, we evaluated associations of the abundance of botflies with: i) nest success; ii) nestling body mass index; iii) nestling tarsus asymmetry. In this regard, we hypothesized that the average number of botflies in nestlings body reduce nest success, reduce nestling body mass index, and increase nestling tarsus asymmetry. Finally, we tested if botfly abundance is predicted by: iv) accumulated rainfall and humidity one week before and one week after hatching date; v) nest wall denseness/openness; vi) vegetation openness; vii) food availability, and viii) host nest density. Relative to these variables, we expected that an increase in the average number of botflies in nestlings' body associated with high accumulated rainfall and humidity, lower nest wall denseness, high vegetation openness, high food availability, and high host nest density.

The description botfly-host interaction patterns improve predictions relative to bird responses to environmental changes. Variation in habitat conditions can lead to increases in parasite abundance, which may contribute to bird extinctions (Bulgarella et al. 2018). Our study expands available information about botfly-host interaction, and enables an in-depth discussion about how this interaction can be influenced by habitat.

Methods

Study site and species

This study was carried out within the University of Brasília campus, in central Brazil (15°45'S; 47°52'W), in an area of 20 ha. The vegetation is classified as Cerrado *sensu stricto* (tropical savanna) with high plant diversity (Aguilar et al. 2008, Assunção and Felfili 2004). We monitored breeding activities of the blue-black grassquit between November to April, among two breeding seasons (2017-2018; 2018-2019). Throughout breeding season, males of blue-black grassquits defend territory and attract mates by performing multimodal displays (Manica et al. 2016a, Manica et al. 2016b). Both sexes build the nest and provide parental care. Nests are small cup-shaped, placed in the forks of shrubs (more frequently) or in dense grass undergrowth (Carvalho et al. 2007, Aguilar et al. 2008). Little is known about botfly infection in this species, but literature reports indicate that blue-black grassquits are infected by the *subcutaneous P. glaucinis* and *P. trinitensis* (Teixeira 1999). Predation is considered the main factor leading to lack of breeding success (Aguilar et al. 2008, Almeida and Macedo 2001, Carvalho et al. 2007, Dias et al. 2010). Cheating behavior, such as extra pair paternity and intraspecific brood parasitism, are observed in blue-black grassquits (Carvalho et al. 2006, Manica et al. 2016a).

Data collection

Nests were searched by taking slow walks throughout grassland fields, performed with a frequency of at least two times a week for in all study area. Nests were checked at intervals of up to three days, until chicks fledged or the nest was lost to predation. Incubation and nestling periods lasted up to 10 days each (Carvalho et al. 2007). If eggs were present in a nest in a given day and on the next day the eggs had hatched, we assumed that hatching occurred on the second day. For nests hatching after a checking interval of two-three days, or found in the nestling period (20 of 180), we estimated hatching day by comparisons with chicks of known age. The disappearance of eggs before hatching or of nestlings before seven days of age was attributed to nest predation. Nestling death due to botfly larvae infestation was assumed when nestlings were found dead in the nest and larvae were found in the nest or nestling. Nest desertion was assumed when parents no longer cared for the eggs (eggs that remained in the nest > 10 days). Whenever eggs were deserted, we collected and opened them to check for development. After this inspection, eggs were classified as "infertile" if no embryo was found, or "death in development" if we found a dead embryo. Nest success was assumed when nestlings disappeared from the nest at or later than 7 days post-hatch and no signs of predation were detected (*ie.* the nest remained intact in the vegetation).

Nestling body condition was recorded up to three times for each nestling, which included mass with a spring scale (Pesola 10g, 0.1g resolution), both left and right tarsus length (digital caliper Mitutoyo 500-196-30B, 0.01 mm resolution), number of larvae and their location on the nestling´s body (we did not identify larvae to the species level). A body mass index was calculated by dividing nestling weight by average tarsus length. In adult grassquits, it has been found that body mass index correlates negatively with intestinal parasite load (Aguilar et al.

2008, Costa and Macedo 2005), social dominance (Santos et al. 2009), and males with a lower body mass index shown trade-offs among display attributes (Manica et al. 2016a, Manica et al. 2016b). Nest body asymmetry was calculated based on the absolute difference between left and right tarsus lengths. The location of larvae was mapped onto the nestling´s body areas: head-neck, wings, legs, and main body (see figure 1); large larvae could occupy more than one area. We did not band nestlings at the nest with leg bands since this could influence natural predation rate by increasing the contrast between nestlings and nest background material.

Climatic data was obtained from the open database provided by the Brazilian metereological institute (Instituto Nacional de Meteorologia - INMET), which provided a regional sampling location less than 10 km from the study site. For each nest, we averaged the daily rainfall and temperature for a period encompassing 14 days, from seven days before and after hatching.

When nests were no longer active, they were collected to determine their aspects of their architecture. After measurements were taken, nests were deposited in the museum collection Coleção Ornitológica Marcelo Bagno, at Universidade de Brasília. We used two variables to characterize nest architecture: nest wall density and nest wall openness. Nest wall density was calculated as the ratio of nest weight to wall volume (mg/mm^3). Nest weight was obtained with a high precision balance (Shimadzu BL320H, 1mg resolution) after the nest was air dried at 75ºC for 24h. Nest wall volume was estimated as the difference between external and internal nest wall volumes. Volume was estimated using the semi-ellipsoid volume formula ($V = 2/3 \times \pi \times a \times$

b × c, where a and b are perpendicular measurements of nest outer/inner diameter and c is height/depth). Nest wall openness was estimated as the average of four measurements of nest wall openness taken on different sides of the nest. A detailed description of the method is provided in Biagolini-Jr and Macedo (2019). In summary, photos of the nest were taken with a white styrofoam ball (50mm in diameter) placed inside the nest chamber. Then, with an image editor software (GIMP version 2.10) we cropped the styrofoam ball image section and converted it to a black and white scale. Using the R package bwimage (Biagolini-Jr 2019), we estimated nest wall openness as the proportion of white pixels relative to total number of image pixels.

We assessed food availability and vegetation density in five spots at distances of 3 m from the nest within two weeks after nestlings fledged. We did not demarcate parental territories, but assume that the sampled spots had a high probability of falling within territories (Aguilar et al. 2008). We estimated the abundance of seed resources by averaging the number of seed inflorescences counted in 50 x 50 cm grids placed at each of the five spots (Manica et al. 2014). Vegetation density was estimated in five plots of 30x100 cm, by adapting the Zehm et al. (2003) method. In summary, a photograph was taken of the vegetation against a panel of 100x100 white cloth placed perpendicularly to the ground on the largest side of the plot. The photograph was converted to a pure black and white image (GIMP version 2.10). Vegetation density was estimated as the proportion of black pixels relative to total number of pixels (Biagolini-Jr and Macedo 2019).

Host nest density was estimated as the number of grassquit nests, within a radius of 50 meters, with clutches that hatched in the period of 10 days before and after nest hatching date at the focal nest being considered. We chose this range, because it encompasses both the nestling period and the botfly pupation period from a possible previously infected nest (Saravia-Pietropaolo et al. 2018). We used the R package geosphere Version 1.5 (Hijmans 2019) to calculate distances between nests.

Data analyses

We performed model fitting by Generalized Linear Mixed Model (GLMM) using the R package glme4 version 1.1 (Bates et al. 2014). GLMM was chosen because it allows us to control non-independence among nestlings as well as multiple measurements from the same nests. Models were fitted to evaluate association of the number of botflies and: nestling survival (model 1), body mass index (model 2), tarsus asymmetry (model 3), climatic condition (model 4), nest morphology (model 5), and habitat parameters (model 6). As we found a direct relationship of tarsal asymmetry and number of larvae (see results - model 3), we additionally tested whether the asymmetry could be explained by the difference in number of larvae on each of the nestlings´s legs (model 7). Model 1 assumes nests as sample units; thus, we averaged the number of botfly larvae from nestlings in the same brood to obtain average nest infection intensity; all other models used nestlings as sample units. Table 1 summarizes global models' formulas and link functions. We used nest identity as a random effect for all models; we were unable to include nestling identity as a fixed effect because we did not band chicks in the nest.

Parental identity could not be used either, since only 25.3% of the sampled nests (19 of 75) had at least one of the parents banded.

We performed mathematical modelling according to (Zuur et al. 2009). We included nestling age and brood size as possible confounding predictors. Exceptionally, we did not include Age in model 1 (nestling success as response variable), because nestlings that survived until a later date had a higher probability of fledging than new nestlings. An age effect on the number of botfly larvae was expected because older nestlings had a longer exposure time for infection. Brood size may have two opposing effects on larvae abundance: it can increase visual and olfactory cues for adult flies to find the nests; on the other hand, once a fly finds the nest, there is a parasite dilution effect that can lead to reduction in the abundance of larvae per chick (Dudaniec et al. 2007). In a preliminary analysis, we checked whether predictors from model 5 and model 6 could be correlated. For model 5, nest wall density and nest wall openness was significantly (Pearson's t test, p-value = 0.02, t = -2.30, df = 163, estimated correlation = -0.18). Thus, we use chose to use only wall openness as predictor in the global model 5. For model 6, food supply was not significantly associated with either nest density (Pearson's t test, p-value = 0.43, t = -0.78, df = 76) or vegetation density (Pearson's t test, p-value = 0.06, t = 1.90, df = 107, estimated correlation = 0.18). Thus, we use all variables as predictor in the global model 6.

The appropriate distribution of response variables was defined by the nature of each response variable (Harrison et al. 2018, Zuur et al. 2009). All predictor variables were scaled to mean 0 and standard deviation 1, as transforming predictor variables improves model performance and interpretation interpretability (Gelman and Hill 2007, Sommet and Morselli 2017).

Model selection was performed by backward stepwise model selection, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model (MAM). We tested interactions among predictor variable by introducing all possible predictor interactions in global model's following by the test of its significance – see Table 1. In order to mitigate bias and increase estimation accuracy, we performed the stepwise reintroduction for parameter estimation (SRPE), and checked each parameter fitting (Hegyi and Garamszegi 2011, Hegyi and Laczi 2015). We confirmed that predictor variables that were not previously removed returned to the model after SRPE. We assumed no evidence to support the tested hypothesis when only age, brood size or no variable (null model) was present in MAM. For MAMs with multiple predictor variables, we checked collinearity with the variance inflation factor (VIF), calculated by vif function from car Package (Fox et al. 2019). We assumed the threshold of 3 as evidence for collinearity (Zuur et al. 2010). We checked model validation by graphic inspection of homogeneity, residuals normality, and independence of each explanatory variable (Zuur et al. 2009). We did not observe overdispersion in models with non-gaussian error structure (Harrison et al. 2018, Zuur et al. 2009).

Results

Across two breeding seasons, we found 180 grassquit nests, containing 423 eggs/nestlings. Only 17 nests had complete success with all chicks fledging, while 11 had partial success (at least one chick fledged). In 12 nests parents deserted the entire clutch, while in 7 nests one or more eggs were abandoned once an offspring from the same clutch fledged. Of these nests where at least some eggs were deserted, 11 eggs were infertile and 8 eggs contained death embryos. Predation was the main factor resulting in breeding failure: 105 nests were lost to predation (53 during the laying or incubation periods; 52 during the nestling period). Additional causes for nest loss included: 3 nests where all nestlings died due to botfly infection; 14 were lost due to human activities (fire or mowing); 5 had the eggs broken by the researcher while handling eggs to collect data of eggs morphology (data not show). Due to logistic problems 13 nests could not be checked for twelve consecutive days (from January-23 to February-03 2019) and were not considered in the final survival calculation. Overall, 52 grassquit offspring fledged, representing a success rate of 15% (n=340), after excluding human-related nest losses. We suggest that cases of partial egg predation and desertion could have three causes: i) occurrence of predation after the first egg was laid; ii) ejection of a parasitic egg; iii) giving up infertile eggs. In one nest, we observed a new egg laid close to the hatching date, and it was then ejected after the other eggs hatched.

We collected data on botfly infection for 164 nestlings from 75 nests. We found botfly infection in 54 (32.9%) nestlings from 29 (38.7%) nests. Among infected nestlings, the average number of observed larvae was 2.95 (range 1-18), with the head-neck being the most affected body area; in fact, 93.10% of infected nests had at least one nestling bearing larvae on this body area. The following most infected areas were: main body, legs and wings, where 72.41%, 55.17% and 37.93% of nests had at least one chick infected in these body areas, respectively.

Data exploration indicated a potential outlier among records. For one nestling, we recorded an increase of more than 10 larvae within 2 days. Although we did not verify the size of the larvae, in this case we noticed the presence of a large number of small-sized larvae and the nestling was breathing with difficulty (panting breath), suggesting that the infection would result in death. However, the nestling disappeared from the nest the following day, and we assumed this was due to predation. Given these characteristics, we chose to drop this record from the modelling analyses.

Our results indicate that, the average number of larvae in nestling bodies do not influence nest success (model 1). Opposing to one prediction, number of larvae was positively related to nestling body mass index ($\beta = 0.012$, SE =0.05, $\chi 2 = 5.12$, df =1, p=0.02 – model 2). As predicted botflies increased tarsal asymmetry ($\beta = 0.398$, SE =0.06, $\chi 2 = 32.12$, df =1, $p<0.01$ – model 3). But this asymmetry is not linked with the difference in number of larvae on each offspring leg (model 7). We did not find evidence that average number of larvae is linked with climatic conditions (model 4) or nest morphology (model 5). The number of larvae was negatively correlated to habitat food availability ($\beta = -0.670$, SE = -0.29, $\chi 2 = 5.71$, df =1, p=0.02 – model 6), which indicated that territories with abundant food resources had offspring with a low number of larvae. Backward stepwise model selection present in supplementary.

Discussion

Information about tropical avian breeding biology is relatively scarce, and little is known about critical nesting parameters, such as clutch size, incubation period and nestling period for the majority of species (Xiao et al. 2016). Although many studies have elucidated relevant aspects of the blue-black grassquit´s breeding biology (summarized in Macedo and Manica 2019), there are several gaps in information. Here we present the first estimation of nesting success taking into consideration the effects of botfly infection on grassquit breeding. The head and neck areas were the most affected body areas, and almost all infected nests had at least one nestling infected in this body region. As the head-neck body region surface is smaller than other body parts, this suggests a non-radom tendency of infection in this body region. We suggest that this is the first point of contact of the adult fly with the nestlings, which most likely occurs when the parents are out of the nest (*ie.* they are not incubating the nestling).

Overall, grasssquit nesting success is low (15%, n=340), even when compared to other Thraupidae species from the same biome, which ranges from a low of 20% - *eg*. coal-crested finch *Charitospiza eucos*ma (Diniz et al. 2013) to a high of 40% lined seedeater *Sporophila lineola* (de Oliveira et al. 2010). Just a fraction (17.7%) of blue-black grassquit nests were infected by botflies, and death as a direct consequence of infection occurred for less than 1% of the nestlings (3 of 340). Furthermore, model 1 indicated that the abundance of larvae does not affect nest success, a result similar to what has been observed for other passerines in the same Cerrado biome (Lopes and Marini 2005). Together, this evidence suggests that botflies have a low impact on grassquit nestling survival, and validates conclusions that predation by vertebrate predators (*eg.* mammals and birds) is the main source of breeding failure (Almeida and Macedo 2001, Carvalho et al. 2007, Dias et al. 2010, Aguilar et al. 2008).

Contrary to our prediction, infected offspring showed a higher body index (Model 2). In the literature, this specific body index is usually associated with a better body condition, since it reflects the accumulation of fat and muscles, which should thus lead to greater fitness (Walsberg, 1988, Ekman and Hake, 1990). Intriguingly, our results for the grassquit appears to contradict this tendency, as infected nestlings had higher body index. As a physiological response to infection, nestlings may be hungrier and perform more begging calls which may lead to higher parental feeding, *ie.* food compensation hypothesis, investment and increased weight of such nestlings (Hurtrez-Boussès et al. 2000, Johnson and Albrecht 1993, Tripet et al. 2002, Tripet and Richner 1997). Alternatively, larvae weight per se could explain the higher body index, given that the weight taken of the nestlings included all larvae. Given the impossibility of parasite removal to assess nestling weight, this hypothesis remains to be tested.

The number of botflly larvae was positively associated with grassquit nestling tarsus asymmetry (Model 3). As far as we know, this is the first record of increased asymmetry for botfly-infected nestlings. Fluctuating asymmetry is a reflection of an individual's inability to buffer developmental disturbances (Lens and Van Dongen 2000, Swaddle and Witter 1997). Thus, body symmetry can reflect developmental homeostasis (Debat and David 2001, Dongen 2006, Klingenberg 2015) and quality of the habitat during the animal´s development (Anciães and Marini 2000, Cuervo and Restrepo 2007, Lens and Eggermont 2008). Body symmetry is positively related to adult body fat (Prentice et al. 2008), and is used as a trait for mate choice (Evans 1993). This suggests that even if grassquit nestlings survive larval infection, it may have a negative consequence later in life. Adults that were infected in the nest may have reduced fitness due to less attractive body traits or a disadvantage in motor performance, which may be a determinant factor for species with sexual display (Manica et al. 2016b). As tarsus asymmetry was not explained by difference in number of larvae on each leg (model 7), we suggest that the effect of the number of larvae on symmetry is due to parasite-induced nutritional stress (Brown and Brown 2002).

Contrary to our expectations, climatic conditions (model 4) and nest morphology (model 5) were not linked to botfly abundance, contradicting a previous study (Antoniazzi et al. 2011, Arendt 2000, Langen and Berg 2016, Manzoli et al. 2013, Nores 1995). Results indicate that humidity and temperature maintenance are not determining factors for larvae abundance. However, one environmental factor, food abundance, was negatively correlated to larvae abundance. This result, in association with the fact that most chicks are infected in the head-neck region, suggests that infections occur when parents are away from the nest and searching for food to feed the offspring. Therefore, by selecting a territory with high food availability, parents can spend more time at the nest, leading to parasite-free and healthier offspring.

In conclusion, botflies did not significantly reduce nest success and had a low impact on nestling survival. The most infected body regions of blue-black grassquit was the head and neck. Parasitic load was positively associated with high body mass index and body asymmetry. Territories with a high food supply contained more parasite-free broods, which could reflect parents spending less time away from the nest. There was no evidence linking botfly abundance with climatic condition and nest morphology.

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Conflict of interest - The authors declare that they have no conflict of interest.

Permits - This survey is part of a project to explore the role of habitat in blue-black grassquit breeding biology. Methods used in this study are in accordance with ethical standards and Brazilian laws. The relevant authorities registered licenses for this project are: Bioethics Committee of Universidade de Brasília license no. 66711/2016; Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) license no. 51639; and Centro Nacional de Pesquisas para Conservação das Aves Silvestres (CEMAVE) license no. 4255).

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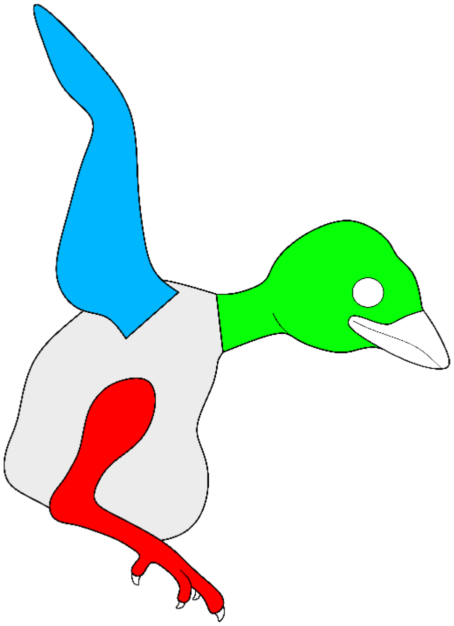
Figure

Figure 1. Representation of four body areas, of blue-black grassquit Volatinia jacarina nestling potentially infected by Philornis sp. The four areas are: head-neck (green), wings (blue), legs (red), and main body (gray).



Tables

Table 1. Summary of hypotheses tested in this study expressed by global models' formulas and link functions.

Model	Formula	Family	Link
Model I	Success ~ BroodSize + AvNBotfly +(1|NestID)	Binomial	logit
Model II	BodyMassIndex ~ Age + BroodSize + NBotfly +(1|NestID)	Gaussian	identity
Model III	ln(TarsusAsymmetry) ~ Age + BroodSize + NBotfly +(1|NestID)	Gaussian	identity
Model IV	NBotfly ~ Age + BroodSize + Temperature*Rainfall+(1|NestID)	Poisson	log
Model V	NBotfly ~ Age + BroodSize + NestWallDensity * NestWallHoles + (1|NestID)	Poisson	log
Model VI	NBotfly ~ Age + BroodSize + Ninflorescence * NestDensity * VegetationDensity + (1|NestID)	Poisson	log
Model VII	TarsusDif ~ Age + BroodSize + BotflyDif	Gaussian	identity

Key

Success: binary response to nest success (0 = fail; 1= success)
BroodSize: brood Size
AvNBotfly: average number of larvae in nestling bodies
Nbotfly: number of larvae in nestling body
BodyMassIndex: nestling weight (g) / tarsus length (mm)
Age: age (days)
TarsusAsymmetry: absolute difference of left and right tarsus (mm)
Temperature: average temperature (ºC) within 20 days before and 10 days after hatching
Rainfall: average rainfall (mm) within 20 days before and 10 days after hatching
NestWallDensity: Nest weight (g)/ nest wall volume (mm3)
NestWallHoles: Proportion of wall nest wall gaps in relation to nest wall material -see Biagolini-Jr & Macedo 2019
Ninflorescence: Average number of seed inflorescences in plot of 50cm2
VegetationDensity: Vegetation Density - see Biagolini-Jr & Macedo 2019
NestDensity: Number of grassquit nests within a radius of 50 meters, that hatching in the period of 20 days before and 10 after nest hatching date
TarsusDif: Length of left tarsus - Length of right tarsus (mm)
BotflyDif: Number of larvae in left leg - Number of larvae in right leg

Table 2. Beta coefficients and significance test of MAM (minimally adequate models) from models that support tested hypothesis.

Model	Minimally adequate models	Coefficients	β	SE	χ2	p
II	BodyMassIndex ~ Age + NBotfly + (1 | NestID)	Age	0.591	± 0.063	71.33	<0.001
		NBotfly	0.012	± 0.057	5.12	0.02
III	ln(TarsusAsymmetry) ~ NBotfly + (1 | NestID)	NBotfly	0.398	± 0.066	32.12	<0.001
VI	NBotfly ~ Age + Ninflorescence + (1 | NestID)	Age	0.807	± 0.158	28.13	<0.001
		Ninflorescence	-0.67	± 0.296	5.71	0.02

Supplementary material

Table S1. Backward stepwise model selection of Model I, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: Survival (0 = fail; 1= success); predictor variables: Brood size (Maximum number of eggs/nestlings found in the nest), AvNBotfly (average number of botfly larvae in the nestlings from the given nest).

Step	Term	Df	AIC	χ^2	p
1	<none>		89.297		
	BroodSize	1	87.444	0.15	0.70
	AvNBotfly	1	87.554	0.26	0.61
	Step 1 conclusion: drop term BroodSize				
2	<none>		87.444		
	AvNBotfly	1	85.646	0.20	0.65
	Step 1 conclusion: MAM = Survival ~ 1 + (1 | NestID)				

Table S2. Backward stepwise model selection of Model II, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: BodyMassIndex (Index of body condition); predictor variables: Age (age in days), BroodSize (maximum number of eggs/nestlings found in the nest), NBotfly (total number of botfly larvae in the given nestling body).

Step	Term	Df	AIC	χ^2	p
1	<none>		300.68		
	Age	1	370.26	71.59	< 0.001
	BroodSize	1	299.09	0.42	0.51
	Nbotfly	1	303.9	5.22	0.03
	Step 1 conclusion: drop term BroodSize				
2	<none>		299.09		
	Age	1	368.43	71.33	< 0.001
	Nbotfly	1	302.22	5.12	0.02
	Step 2 conclusion: MAM = BodyMassIndex ~ Age + NBotfly + (1 | NestID)				

Table S3. Backward stepwise model selection of Model III, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: TarsusAsymmetry (Natural logarithm of left and right tarsus difference); predictor variables: Age (age in days), BroodSize (maximum number of eggs/nestlings found in the nest), NBotfly (total number of botfly larvae in the given nestling body).

Step	Term	Df	AIC	χ^2	p
1	<none>		558.25		
	Age	1	556.92	0.67	0.41
	BroodSize	1	557.24	0.99	0.32
	Nbotfly	1	584.4	28.14	< 0.001
	Step 1 conclusion: drop term Age				
2	<none>		556.92		
	BroodSize	1	555.81	0.89	0.34
	Nbotfly	1	587.92	33.00	< 0.001
	Step 1 conclusion: drop term BroodSize				
3	<none>		555.81		
	Nbotfly	1	585.94	32.12	< 0.001
	Step 3 conclusion: MAM = ln(TarsusAsymmetry) ~ NBotfly + (1 |NestID)				

Table S4. Backward stepwise model selection of Model IV, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: NBotfly (Total number of botfly larvae in the nestling body); predictor variables: Age (age in days), BroodSize (maximum number of eggs/nestlings found in the nest), Rainfall (average the daily rainfall within 7 days before and after hatching), Temperature (average the daily temperature within 7 days before and after hatching).

Step	Term	Df	AIC	χ^2	p
1	<none>		428.72		
	Age	1	450.03	23.32	< 0.001
	BroodSize	1	427.93	1.21	0.27
	Rainfall:Temperature	1	427.13	0.41	0.52
	Step 1 conclusion: drop term Rainfall:Temperature				
2	<none>		427.13		
	Age	1	449.03	23.90	< 0.001
	BroodSize	1	426.15	1.01	0.31
	Rainfal	1	427.31	2.18	0.14
	Temperature	1	426	0.87	0.35
	Step 2 conclusion: drop term Temperature				
3	<none>		426		
	Age	1	447.92	23.92	< 0.001
	BroodSize	1	424.72	0.72	0.40
	Rainfal	1	425.43	1.43	0.23
	Step 3 conclusion: drop term BroodSize				
4	<none>		424.72		
	Age	1	447.07	24.34	< 0.001
	Rainfall	1	424.24	1.51	0.22
	Step 4 conclusion: drop term Rainfall				
5	<none>		424.24		
	Age	1	445.43	23.20	< 0.001
	Step 5 conclusion: MAM = NBotfly ~ Age + (1 | NestID)				

Table S5. Backward stepwise model selection of Model V, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: NBotfly (Total number of botfly larvae in the nestling body); predictor variables: Age (age in days), BroodSize (maximum number of eggs/nestlings found in the nest), NestWallHoles (proportion of holes in the nest wall, when contrasted with a white background).

Step	Term	Df	AIC	χ^2	p
1	<none>		382.69		
	Age	1	402.15	21.47	< 0.001
	BroodSize	1	382.97	2.28	0.13
	NestWallHoles	1	381.33	0.64	0.42
	Step 1 conclusion: drop term NestWallHoles				
2	<none>		381.33		
	Age	1	401.83	22.50	< 0.001
	BroodSize	1	381.34	2.01	0.16
	Step 2 conclusion: drop term BroodSize				
3	<none>		381.34		
	Age	1	402.19	22.85	< 0.001
	Step 3 conclusion: MAM = NBotfly ~ Age + (1 | NestID)				

Table S6. Backward stepwise model selection of Model VI, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: NBotfly (Total number of botfly larvae in the nestling body); predictor variables: Age (age in days), BroodSize (maximum number of eggs/nestlings found in the nest), Ninflorescence (average number of seed inflorescences in 5 plots around nest), NestDensity (number of grassquit nests within a radius of 50 meters, from period of 10 days before and after nest hatching date), VegetationDensity (proportion of white pixels from a black and white picture from vegetation around nest).

Step	Term	Df	AIC	χ^2	p
1	<none>		400.22		
	Age	1	429.24	31.01	< 0.001
	BroodSize	1	399.07	0.85	0.36
	Ninflorescence:NestDensity:VegetationDensity	1	398.22	< 0.001	>0.999
	Step 1 conclusion: drop term Ninflorescence:NestDensity:VegetationDensity				
2	<none>		398.22		
	Age	1	427.46	31.23	< 0.001
	BroodSize	1	397.13	0.90	0.34
	Ninflorescence:NestDensity	1	401.29	5.07	0.02
	Ninflorescence:VegetationDensity	1	396.35	0.12	0.73
	NestDensity:VegetationDensity	1	397.8	1.58	0.21
	Step 2 conclusion: drop term Ninflorescence:VegetationDensity				
3	<none>		396.35		
	Age	1	425.65	31.30	< 0.001
	BroodSize	1	395.17	0.83	0.36316
	Ninflorescence:NestDensity	1	399.29	4.95	0.03
	NestDensity:VegetationDensity	1	396.77	2.42	0.12
	Step 3 conclusion: drop term BroodSize				
4	<none>		395.17		
	Age	1	425.06	31.89	< 0.001
	Ninflorescence:NestDensity	1	398.01	4.84	0.03
	NestDensity:VegetationDensity	1	395.73	2.56	0.11
	Step 4 conclusion: drop term NestDensity:VegetationDensity				
5	<none>		395.73		
	Age	1	424.82	31.09	< 0.001
	VegetationDensity	1	396.02	2.29	0.13
	Ninflorescence:NestDensity	1	398.09	4.36	0.04
	Step 5 conclusion: drop term VegetationDensity				

6	<none>		396.02		
	Age	1	423.49	29.46	< 0.001
	Ninflorescence:NestDensity	1	397.82	3.80	0.051
	Step 6 conclusion: drop term Ninflorescence:NestDensity				
7	<none>		397.82		
	Age	1	423.84	28.01	< 0.001
	Ninflorescence	1	401.54	5.72	0.02
	NestDensity	1	395.84	0.01	0.91
	Step 7 conclusion: drop term NestDensity				
8	<none>		395.84		
	Age	1	421.97	28.13	< 0.001
	Ninflorescence	1	399.54	5.71	0.02
	Step 8 conclusion: MAM = Age + Ninflorescence + (1 | NestID)				

Table S7. Backward stepwise model selection of Model VII, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: TarsusDif (difference of left and right tarsus length) predictor variables: BotflyDif (difference in number of larvae on left and right body sides).

Step	Term	Df	AIC	χ^2	p
1	<none>		159.42		
	Age	1	161.61	4.19	0.04056
	BroodSize	1	161.78	4.36	0.03677
	BotflyDif	1	157.5	0.09	0.76718
	Step 1 conclusion: drop term BotflyDif				
2	<none>		157.5		
	Age	1	159.96	4.46	0.03472
	BroodSize	1	159.91	4.41	0.03574
	Step 2 conclusion: MAM = TarsusDif ~ Age + BroodSize + (1 | NestID)				

CAPÍTULO 3

The role of predation risk in tropical passerine breeding

Este manuscrito será submetido ao periódico *Journal of Animal Ecology* após a apreciação pela banca examinadora. Portanto, este segue o padrão de referências bibliográficas do periódico.

Abstract

Predation is the main factor that determines nest success among birds. There is growing evidence that predation risk can induce changes in breeding behavior. Using blue-black grassquits *Volatinia jacarina* as our model, we tested whether predation risk can induce variation in cheating behaviors (extra pair paternity and intraspecific brood parasitism), brood size, offspring sex ratio and body mass index. Over two breeding seasons, we experimentally manipulated the birds' perception of predation risk by exposing them to predator and non-predator models in two independent areas, and used a third area as a secondary control (*ie.* absence of model exposure). In parallel with the experimental manipulations, we evaluated the physiological stress response of birds by determining their heterophils to lymphocytes blood ratio (H/L-ratio) levels. Our results did not show any variation in cheating behaviors, brood size or body mass index among experimental groups. We found that females exposed to the harmless species model produced more female offspring and that only birds in the secondary control area showed a variation in their H/L-ratio, with increasing levels of stress across the breeding season. We conclude that associations that have been proposed between predation risk and extrapair fertilization hinge upon the bird's perception of danger. Additionally, our results suggest that grassquit females can vary brood sex ratio according to environmental contexts. Our findings extend awareness about how researcher presence may increase levels of physiological stress in the study animal.

Introduction

Nest predation is a determinant factor for bird breeding success, shaping several breeding characteristics, such as choice of nest site (Eggers et al. 2006), brood size (Martin et al. 2000), incubation and nestling periods (Massaro et al. 2008), and parental care (Ghalambor and Martin 2000). Although predation influences life history evolution, nest success is context dependent and unpredictable for parents. For instance, several bird species increase their fitness by reuse of successful nests, but build a new nest when the previous one is depredated (*eg.* star-throated antwren *Rhopias gularis*, de Lima and Roper 2016; spotted antbirds *Hylophylax naevioides*, Styrsky 2005; vermilion flycatchers *Pyrocephalus rubinus*, Ellison 2008; mao *Gymnomyza samoensis*, Stirnemann et al. 2016). When a breeding bird is exposed to a nest predator, two types of response are expected: i) an immediate response, in an attempt to avoid predation of the current nest; and ii) a long-term response, intended to improve performance in future breeding attempts.

Skutch (1949) proposed that predators use parental movements to locate nests. Thus, when faced with an imminent risk of nest predation the main expected response of the parents should be to avoid nest attendance. For example, when exposed to predator models, male sexually ornamented blue-black grassquits *Volatinia jacarina* become more discreet and reduce nest visitation rates (de Moraes 2019). When predation risk is constant, birds can also decrease predator attraction and encounter rates by reducing their activities within the habitat (Abbey-Lee et al. 2018). However, continuous use of this strategy entails difficulty in obtaining food, which can lessen body condition (Abbey-Lee et al. 2016, Mathot et al. 2016) and breeding performance by reducing brood size and/or brood survival (Santema et al. 2019b).

Avoiding predation can be complex, given high predator diversity (Menezes and Marini 2017). Predation can occur any time of day (Ribeiro-Silva et al. 2018), and predators can use olfactory (Whelan et al. 1994, Biagolini-Jr and dos Santos 2018, Mihailova et al. 2018, Perrella et al. 2019), thermal (Weatherhead and Blouin-Demers 2004, Stake et al. 2005), and auditory (Rice 1982, Halupka 1998, Briskie et al. 1999). Even small pieces of nest material or new-cavity excavation can be used by predators to find nests (Wiebe et al. 2007). Thus, nest predation is almost inevitable in areas with a high diversity of predators. As a consequence, birds from tropical areas have very low breeding success (Skutch 1966, Skutch 1985): 8% for dusky antbirds *Cercomacra tyrannina* (Morton and Stutchbury 2000) and 14% for western slaty antshrikes *Thamnophilus atrinucha* (Roper 2005).

One of the main consequences of a monogamous mating strategy in areas with high predation risk is the high probability of losing all offspring in a single predation event. Cheating behavior, which includes extra pair paternity (EPP) and intraspecific brood parasitism (IBP), can increase individual fitness by spreading offspring across several nests, thus increasing the probability that at least one or a few offspring survive to fledging (Yom-Tov 1980, Brennan 2012). There is a growing number of studies that focus upon this hypothesis. One study of a natural population of great tits *Parus major* found a positive association between nest predation rates and EPP (Yuta and Koizumi 2016). In two other studies, however, predator playbacks did not result in an overall increase in EPP rates (great tits: Abbey-Lee et al. 2018; blue tits *Cyanistes caeruleu*: Santema et al. 2019a). However, the same blue tit population, when exposed to predator models instead of playbacks, showed increased EPP rates (Santema et al. 2019b). The introduction of predator models also increased investment in extra pair offspring in tree swallows *Tachycineta bicolor* (Hallinger et al. 2019). Thus, these studies suggest that birds interpret predator risk as being higher when predator models instead of acoustic cues.

Predation risk can also be considered within the context of sex allocation theory. This theory predicts that in specific ecological, physiological or social conditions, females should bias reproductive investment towards either sex in the offspring, depending upon the potential fitness gains (Trivers and Willard 1973, Frank 1990). This concept has been successfully demonstrated for a number of bird species (*eg.* Dowling and Mulder 2006, Neto et al. 2011, Bowers et al. 2014). For polygamous species, females in poor body condition should benefit by increasing investment in the production of more female than male offspring, since the latter may require a better nutritional condition to achieve breeding. In another context, in species where males perform sexual displays and/or have conspicuous plumage, thus facing increased predation risk, male offspring represent the riskier investment, and females should drive brood sex ratio toward female offspring.

In this study we asked whether predation risk can influence breeding parameters of a tropical bird that performs sexual displays. We used the Neotropical blue-black grassquit as our model for several reasons. First, it occurs across a broad geographic range and can be expected to present high behavioral plasticity. Additionally, predation risk is an important element to consider in terms of impact upon breeding performance, as predation of eggs and nestlings is very high (~70% Aguilar et al. 2008). Previous studies have shown that sexual displays elicit higher predation for both nests (Dias et al. 2010) and adults (de Moraes et al. 2019). We thus hypothesized that blue-black grassquits in areas with higher predation risk would exhibit: (1) reductions in body condition and brood size; (2) increased rates of EPP and IBP; and (3) offspring sex ratio favoring more female offspring.

Methods

Study site and species

We carried out this study from December to March across two breeding seasons (2015-2016 and 2016-2017) within the University of Brasilia campus, in Brasilia, Brazil (15°45'S; 47°52'W; altitude ca 1000m). The vegetation in the study site is Cerrado *sensu stricto* (tropical savanna) with a high diversity of woody trees (Assunção and Felfili 2004) and invasive grasses (*eg. Brachiaria* sp. and *Melinis minutiflora*). The average daily temperature was 21.4° C (minimum 12.1 ° C, maximum: 29.1 ° C), average annual rainfall was 1459.6 mm (minimum: 1157.1 mm, maximum: 1801.3 mm) (INMET 2019) and the Köppen-Geiger climate classification is Aw (Kottek et al. 2006, Peel et al. 2007).

The blue-black grassquit is a species commonly found in grasslands in the Neotropical region. In central Brazil, where this study was conducted, there is an increase in its population between November to April, associated with the rainy season. During this period males molt to a blue-black iridescent nuptial plumage. During the non-breeding season males and females have a light brown plumage coverage (Marcondes-Machado 1987, Moreno-Palacios et al. 2013). Throughout the breeding season males perform multimodal displays across most of the day (Carvalho et al. 2007) from within the limits of their small territories (13.0 to 72.5 m^2; Almeida and Macedo 2001). The male display comprises a short vertical leap (average height of approximately 20 cm (Manica et al. 2016b) initiated from a perch, accomplished through a variable number of wingbeats, and synchronized with a strident vocalization (Manica et al. 2014, Manica et al. 2016a). Displays are performed on average 14 times per minute (Chapter 1) and are assumed to be energetically costly (Manica et al. 2016b).

Grassquits build a small, cup-shaped nest in shrubs or in dense grass undergrowth. Nest site selection is based on microhabitat complexity (Aguilar et al. 2008), and nests are spatially aggregated, which leads to a local increase in population density and predation events (Dias et al. 2009). Both sexes build the nest and provide parental care (Carvalho et al. 2007), but extrapair paternity is exceptionally high (Carvalho et al. 2006, Manica et al. 2016a). Predation is considered the main cause of nest loss (Almeida and Macedo 2001, Carvalho et al. 2007, Dias et al. 2010).

Experimental design

During the two breeding seasons of the study, we used four independent areas of approximately 5 ha each. Throughout each season, we introduced taxidermized models at four random spots in two areas, three times weekly. One area received a taxidermized predator model (see details below), the guira cuckoo *Guira guira*, while the second area received a model of a nonpredator harmless species, the sayaca tanager *Tangara sayaca*. A third area was included in the experimental protocol, but without the introduction of any model. To avoid area-related bias, we swapped the two areas that received models in the second breeding season (Figure 1). We chose a guira cuckoo as our predator model based on previous experience that showed this species to be a predator of both adults and nestlings (see de Moraes et al. 2019). We exposed the models attached to rods at a height of 180 cm while broadcasting playback vocalizations with loudspeakers calibrated at 70 dB (SEW® 2310 SL). The playbacks consisted of 30 s of vocalization of the model species (predator or nonpredator) intercalated with 30 s silence during a 15-min bout. The recordings were downloaded from the Xeno-Canto acoustic library collection (identification codes XC286598 and XC38498).

We captured adults with mist nets from 0530 h to 1100 h. Birds were marked with unique combinations of plastic color bands and one numbered aluminum band supplied by the Brazilian Bird Banding Agency (CEMAVE/ICMBio). To avoid bias in the banding routine, we followed the protocol described by Biagolini-Jr and Macedo (2020), using the GenTag R package (Biagolini-Jr 2019). From each bird, we collected morphological measures following Roos

(2010). Bird weight was obtained with a spring scale (dynamometer Pesola 10g, 0.1g resolution), and tarsus length was estimated as the average length of left and right tarsi (calipers Mitutoyo 530-312B-10, 0.02 mm resolution). To assess body condition, we calculated a body mass index by dividing bird mass by tarsus length.

In the 2016-2017 breeding season we assessed the effectiveness of the predator model by evaluating physiological stress, based on the ratio of blood heterophils to lymphocytes, *ie.* H/L ratio (Gross and Siegel 1983, Ots et al. 1998, Hõrak et al. 2002; reviewed in Maxwell 1993). We collected 50 µl of blood by brachial venipuncture (Owen 2011) from adults in all study areas one week before introducing the models and also some 50 days later (*ie.* middle of breeding season). We used a drop of blood to prepare blood smears, which were air dried and fixed in absolute methanol (Caetano et al. 2014). Cell counts were performed by a commercial laboratory (Santé Laboratório, Brasilia-DF).

We searched for nests by taking slow walks through the fields and inspecting herbaceous vegetation. Whenever a nest was found, we marked its location with a GPS (Garmin 62ST). Because of the high natural predation observed for grassquits in the study area, we collected the eggs after a minimum of three days of incubation, and from these we obtained tissue samples from embryos and extracted DNA for the genetic analyses. When a nest was found during the laying phase, we used the egg flotation test (Westerskov 1950, Mabee et al. 2006) to determine its incubation stage. A previous study determined that grassquit eggs float 72 hours after being laid (Paneczko 2016); thus, we only collected eggs when they floated during the test. For nests found in the nestling period, we collected blood samples using the same procedure applied for adults. Embryo tissue and blood samples were stored in 1.5 ml tubes in 100% ethanol and refrigerated at 4 °C.

Genetic Techniques

We evaluated the occurrence of EPP and IBP in nests by using molecular markers to estimate kinship among offspring. We assumed that broods containing only full siblings (FS) resulted from monogamous mating of the social parents. We considered that EPP resulted in broods containing half siblings (HS). Finally, cases of IBP resulted in broods of genetically unrelated (UR) offspring. We assumed that both female infidelity and quasi-parasitism can be interpreted as EPP (Carvalho et al. 2006, Manica et al. 2016a). Our method does not allow to estimate cheating behavior in nests with only one nestling, or EPP and IBP simultaneously in nest with two nestlings. Therefore, we do not include these cases in the statistical analysis.

We extracted DNA using the QIAGEN® DNeasy Blood & Tissue Kit and genotyped individuals using a set of 15 microsatellites previously tested by Manica et al. (2016a; see Table 1). We performed amplifications using fluorescently labeled primers and PCR products were read in an automated sequencer (BRC Core Laboratory Center, Cornell University). Allele sizes were scored using the software Geneious 11.1.5. We assessed the quality of the genotypic dataset by calculating allelic diversity, observed (HO) and expected heterozygosity (HE), probability of heterozygosity deficit, probability of Hardy-Weinberg equilibrium, exclusion probability for the first and second candidate parent, probability of identity, and probability that the set of loci could not exclude an unrelated pair. All measures were calculated using software Cervus 3.0 (Kalinowski et al. 2007). As expected, we detected a high allelic diversity among the set of 15 microsatellites (Manica et al. 2016a), and both probability of identity and probability that the set of loci could not exclude an unrelated pair were lower than 0.001.

We assessed offspring kinship first by estimating the more likely relationship between the nestlings with the ML-Relate software (Kalinowski et al. 2006). Whenever ML-Relate suggested that two siblings showed parent-offspring like relation (19 of 186), we assumed a FS relation (*ie.* both parent-offspring and FS relations have an *r* of 0.5). In a second step we confirmed the relationship by testing the hypotheses of FS or HS relationships versus a null hypothesis of UR using the Kingroup Software (Konovalov et al. 2004). These two steps confirmed 95% (178 of 186) of relationships. For the remaining comparisons (n=8), we calculated the triadic likelihood relatedness estimator (Wang 2007) index with a 95% confidence using the Related R Package (Pew et al. 2015). We improved accuracy of the index by estimating allelic frequencies by sampling one individual from each nest, and taking the average of the observed frequencies in 1000 iterations. For all relationships, triadic index indicated FS status, because the index estimator range contained 0.5 (expected for FS) and excluded values bellow 0.25 (expected for HS or U).

Molecular sexing was performed according to the Fridolfsson and Ellegren (1999) protocol. The PCR reaction was performed with 10 μL final volume, containing 1 μL of 10x

Buffer, 25 mM MgCl2, 10 mM DNTp, 0.2 µM of 2550F / 2718R primers, Taq (Sigma) 2.5 U/µl, and 5.7 µL H2O, and 2 µL DNA sample (50ng/µl). The reactions were performed in a thermocycler programmed as follows: 94°C for 3 minutes, 41 cycles of 94°C for 30 seconds, 41°C for 30 seconds, 72°C for 40 seconds, and 72°C for 5 minutes. PCR products were read on 3% agarose gel, with ethidium bromide.

Statistical analyses

We tested for differences in the H/L ratio and body mass index before and after introduction of the models with a Student *t*-test. We evaluated differences between brood sizes under different experimental treatments using ANOVA. In order to test if the occurrence of EPP and IBP (binary response variables) could be predicted by the experimental treatments, we fitted Generalized Linear Models (GLM), with a binomial error structure, using the occurrence of EPP or IBP as response variables. We included brood size as a cofactor, since larger broods are more likely to have at least one EPP or IBP offspring. Model selection was performed by backward stepwise model selection, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model (MAM). We assumed no evidence to support the tested hypothesis when only brood size or none of the variables (null model) was present in the MAM. To assess the effect of treatment on offspring sex determination, we used a one sample *t*-test to test if sex ratio differed from the expected male:female sex ratio of 0.5.

Results

In the two breeding seasons and across the three treatment categories, we found 181 grassquit nests (control = 79; non-predator = 45; predator = 57), containing 397 eggs/nestlings (average brood size = 2.19, range = 1-6). We extracted DNA samples from 250 embryos/nestlings from 109 nests (control = 43; non-predator = 27; predator = 39). Unsampled nests resulted from natural predation that occurred in the interval between finding the nest and our sampling opportunity (egg maturation). Since, nests with two offspring could not be EPP and IBP at the same time, when a case of cheating behavior was detected for these nests, we removed it from the estimation of the other cheating behavior. After excluding these nests, and nest with only one offspring (n = 7), we found evidence of EPP in 29 of 95 (26.3%) broods, and evidences of IBP in 14 of 94 (14.89%).

We found an increase in the H/L ratio between the beginning and middle of the breeding season in birds from the control area ($t = -4.21$, $df = 26.37$, $p < 0.001$). No difference was detected during the same interval for the two experimental treatments (non-predator: $t = -0.20$, $df = 49.78$, $p = 0.84$; predator: $t = -0.35$, $df = 23.85$, $p = 0.73$). We found no significant differences in body condition among the three treatments (control: $t = -0.84$, $df = 37.36$, $p = 0.41$; non-predator: $t = -1.69$, $df = 64.27$, $p = 0.10$ predator: $t = 0.87$, $df = 35.05$, $p = 0.39$). Additionally, there were no significant differences in brood size among treatments ($F = 0.15$, $df = 2$, $p = 0.87$) or in EPP and IBP levels (see model selection in the supplementary material). Finally, we found that females exposed to the non-predator models produced broods that deviated from the expected 0.5 sex ratio, favoring a higher production of female offspring (observed sex ratio = 0.33, t = -2.57, df = 53, p-value = 0.01). There were no differences from the expected ratio among broods in the control (observed sex ratio = 0.49, t = 0.10, df = 90, p-value = 0.91) and predator exposed (observed sex ratio = 0.57, t = 1.38, df = 88, p-value = 0.17) groups.

Discussion

Although several studies explore immediate behavioral responses to nest predation risk, little is known about how perception of predation risk influences bird reproduction. In this study, we investigated the prediction that exposure to predator models could reduce body mass index and brood size, increase cheating behavior (EPP and IBP), and change nestling sex ratio in the blue-black grassquit. Contrary to our expectations, we did not detect significant variations in body mass index, brood size, or cheating behavior for birds in the three treatment categories. Additionally, differing from our prediction, females exposed to harmless non-predator models produced more female offspring, and there was no variation in brood sex ratio when birds were exposed to predator models or in a control area without model exposure.

These results puzzled us, but the corresponding data about stress levels of the birds (H/L ratio) provides a possible explanation. Our results show that only birds from the control area devoid of model introduction had changes in their H/L-ratio between the beginning and end of the breeding seasons. This suggests primarily that birds in the areas where the models were introduced were less stressed than those in the area where no manipulation occurred. To understand this scenario, we found it necessary to examine precisely what the H/L ratio can indicate about stress and how birds might interpret predation risk.

The birds' stress response, as interpreted through the H/L-ratio, reflects social and environmental disturbances (Gross and Siegel 1983), as well as the type and duration of the threat (Maxwell 1993). In a past experiment, captive blue-black grassquits exposed to predator versus harmless vocalizations showed no differences in body condition. However, when exposed to predator playbacks individuals had a decrease in the H/L-ratio while those in the control group exhibited an increase in the H/L-ratio (Caetano et al. 2014). Another study, this time with free-living grassquits, showed that individuals had an increase in H/L-ratio by the end of the breeding season (de Lima 2017). Our results were similar in this regard, since the birds in our control group (devoid of any type of experimental manipulation) exhibited an increase in H/L-ratio. Thus, we can assume that the continuous exhibition of predator and non-predator models induces constant H/L ratios across the breeding season.

Two alternative hypotheses can explain our results, based on these patterns. First, the constant exposure to a stressor can trigger a second-phase stress response unique to birds (Maxwell 1993, Maxwell and Robertson 1998, Caetano et al. 2014) Thus, the lack of an increase in H/L-ratio among birds that were exposed to the models (both predator and non-predator) could indicate that birds sustained a high stress response during the experiment (regardless of model type). This is because both models could involve high stress if: (i) grassquits interpret both models as potential predators; (ii) noise pollution caused by the playbacks was a source of stress

(Gravolin et al. 2014); or (iii) the nature of our experimental design, with the frequent presence of the researcher in the areas that received the models induced higher stress.

A second explanation for our results could be that birds in the areas that received the models had no increased levels of stress. This could happen if: (i) the number of natural predators decreased or they were excluded in these areas due to playback noise pollution (Francis et al. 2009, Grendelmeier et al. 2016); (ii) natural predators were more attentive to models than to live birds, decreasing the level of real threat to the birds; and (iii) in the presence of models, high quality males avoided displaying and defending territories (de Moraes et al. 2019), driving local population stress to lower levels. Our methodology does not allow us to distinguish which of these possible factors explain the observed patterns. Future studies should combine the evaluation of an immediate behavioral response to models and/or immediate physiological stress response (*ie.* corticosterone) with a long-term physiological stress indicator (such as response in H/L-ratio).

This is the first study to test the effect of predation risk on EPP and IBP rates for a tropical bird population, where predation is significantly more intense than in temperate areas (Skutch 1966, Skutch 1985, Martin et al. 2000, Marini 2017, Townsend et al 2018). Despite the unexpected result related to variation in the H/L-ratio, our study provides an innovative perspective by examining the effects of predation risk upon EPP and IBP while taking into account a measure of stress among experimental groups. This is essential because the absence of a method to evaluate the stress response to predation risk can make interpretation of the results more difficult. For instance, Yuta and Koizumi (2016) show that natural predation rates are correlated to EPP intensity. However, it is impossible to distinguish if EPP occurs due to high predation risk or because birds in low-quality habitats have to forage more actively, making nest detection easier for predators (Skutch 1949, Martin et al. 2000). In blue tits, for instance, the experimental use of predator versus harmless playbacks does not increase EPP levels (Santema et al. 2019a), however the same population exposed to predator models shows increased EPP rates (Santema et al. 2019b). On the other hand, predator playbacks influence the number of fledglings (Santema et al. 2019a). Such results raise the question of why blue tits only alter EPP levels when exposed to models, if both methods influenced some aspect of behavior. One possible explanation is that different experimental protocols (*eg.* model, playback or both) used in various studies and for different species may affect the natural predator's behavior differently, in which case the prey response could be modulated by changes in other species' behavior (predators or even competitors). Thus, we suggest that future studies that evaluate behavioral responses using model exposure of any type should consider their possible influence at the community level.

Our results show that grassquit females can change brood sex ratio in relation to environmental context. The fact that the groups that received predator and harmless models showed similar stress levels but differed in offspring sex ratio is puzzling. First, we could consider that the areas used for the non-predator models were of low quality. However, grassquit brood size is directly linked to food supply (Dias and Macedo 2011), and since there were no differences in brood size among experimental groups, we believe that all areas had similar food availability. We suggest that the difference in sex ratios may reflect this species' response to social or ecological variables we did not measure, such as interactions with other species in the area or vegetation structural complexity, which confers a degree of concealment in the face of predation risk.

In conclusion, there was no evidence that predation risk induces variation in body mass index, brood size, and cheating behavior. Grassquit females can bias brood sex ratio in relation to environmental context. Differences in stress levels can be induced by a model exposure, regardless of the type of model. We suggest that future studies that evaluate effects of predation risk on breeding behavior should take into account not only the prey species' behavior, but also potential predators and competitors, and also consider both immediate and long-term stress responses.

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Conflict of interest - The authors declare that they have no conflict of interest.

Permits - Methods used in this study are in accordance with ethical standards and Brazilian laws. The relevant licenses for this project are: Bioethics Committee of Universidade de Brasília license no. 66711/2016; Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) license no. 51639; and Centro Nacional de Pesquisas para Conservação das Aves Silvestres (CEMAVE) license no. 4255.

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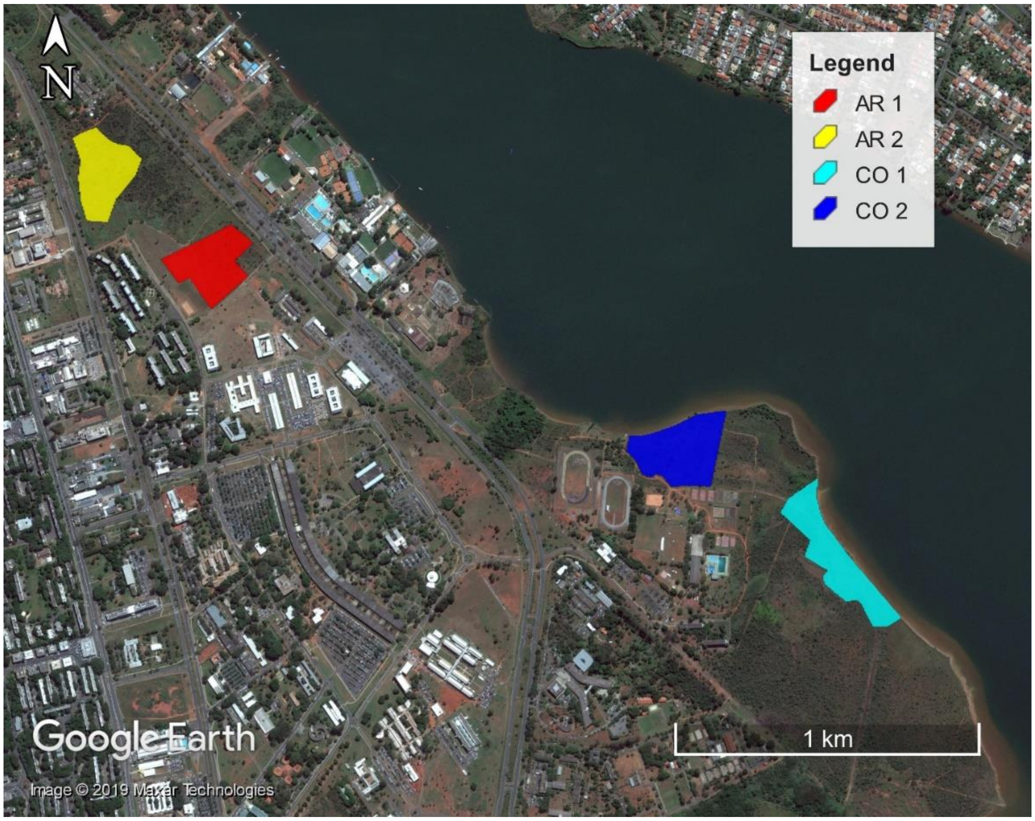
Figure

Figure 1. Study areas used for experimental test of predation risk on Blue-black grassquit *Volatinia jacarina*. The study was conducted within the University of Brasilia campus, in Brasilia, Brazil (15°45'S; 47°52'W), in four areas across two breeding seasons. In the first breeding season (2015-2016), areas CO1 and CO2 received a predator and a harmless model (respectively), and area AR1 was used as a control area. In the second breeding season (2016-2017) areas AR1 and AR2 received a predator and a harmless model (respectively), and area CO2 was used as a control area. All areas had similar vegetation diversity (see Assunção and Felfili 2004), and the sizes of the areas were approximately: AR1 = 4.6 ha, AR2 = 4.6 ha, CO1 = 5.4 ha, CO2 = 5.5 ha (Image source: Google Earth).



Table

Table 1. Characteristics of 15 microsatellite loci tested for kinship analyses. Calculation of all parameters performed in Cervus 3.0 (Kalinowski et al. 2007). Hobs = observed heterozygosity, HExp = expected heterozygosity, NE-1P = Average non-exclusion probability for one candidate parent, NE-2P = Average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex, HW = significance of deviation from Hardy-Weinberg equilibrium. ND = not tested, NS = not significant, * = significant at the 5% level.

Locus	Number of alleles	HObs	HExp	NE-1P	NE-2P	HW	Null allele frequency
Breading season 2015-2016							
VJE5	20	0.855	0.921	0.29	0.17	ND	0.0341
VJJ13	13	0.892	0.872	0.414	0.26	NS	-0.0189
TG11-011	7	0.675	0.736	0.669	0.492	NS	0.0479
GF11	8	0.59	0.569	0.827	0.679	NS	-0.0212
GF12	19	0.843	0.891	0.356	0.217	NS	0.0274
TG03-098	5	0.602	0.534	0.855	0.727	NS	-0.0787
GF16	4	0.06	0.059	0.998	0.97	ND	-0.0069
TG04-061	4	0.422	0.392	0.923	0.791	ND	-0.0463
TG13-017	11	0.771	0.767	0.607	0.423	NS	-0.0086
GF14	8	0.229	0.246	0.968	0.861	ND	0.068
TG02-088	5	0.578	0.485	0.881	0.764	NS	-0.0991
GF01	8	0.361	0.439	0.896	0.746	NS	0.117
TG03-002	5	0.723	0.681	0.74	0.57	NS	-0.03
TG22-001	6	0.494	0.55	0.845	0.717	NS	0.0477
TG01a48	8	0.831	0.765	0.637	0.459	NS	-0.0465
Breading season 2016-2017							
VJE5	25	0.904	0.929	0.26	0.15	ND	0.0115
VJJ13	16	0.79	0.864	0.426	0.269	NS	0.046
TG11-011	11	0.743	0.715	0.692	0.52	NS	-0.0193
GF11	10	0.575	0.561	0.834	0.699	NS	-0.0182
GF12	19	0.868	0.904	0.324	0.193	NS	0.0204
TG03-098	4	0.635	0.561	0.841	0.71	NS	-0.073
GF16	8	0.138	0.132	0.991	0.93	ND	-0.026
TG04-061	7	0.473	0.429	0.899	0.743	NS	-0.0719
TG13-017	12	0.701	0.769	0.6	0.417	NS	0.0529
GF14	11	0.257	0.244	0.968	0.861	ND	-0.0439

TG02-088	3	0.533	0.514	0.869	0.769	NS	-0.0149
GF01	10	0.371	0.41	0.906	0.753	NS	0.0634
TG03-002	5	0.557	0.593	0.815	0.661	NS	0.0274
TG22-001	6	0.479	0.494	0.875	0.752	NS	0.02
TG01a48	8	0.82	0.754	0.636	0.454	NS	-0.0447

Supplementary material

Table S1. Backward stepwise model selection of Model I, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: Extra Pair Paternity (EPP); predictor variables are: brood size (BZ) and experimental group (EG).

Step	Term	Df	AIC	χ^2	p
1	<none>		116.92		
	BZ:EG	2	114.11	1.18	0.55
	Step 1 conclusion: drop term BZ:EG				
2	<none>		114.11		
	BZ	2	114.40	2.30	0.13
	EG		111.30	1.19	0.55
	Step 2 conclusion: drop term EG				
3	<none>		111.30		
	BZ	1	111.50	2.21	0.14
	Step 3 conclusion: MAM = EPP ~ 1 + ε				

Table S2. Backward stepwise model selection of Model II, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: Intraspecific Brood Parasitism (IBP); predictor variables are: brood size (BZ) and experimental group (EG).

Step	Term	Df	AIC	χ^2	p
1	<none>		84.66		
	BZ:EG	2	80.83	0.17	0.92
	Step 1 conclusion: drop term BZ:EG				
2	<none>		80.83		
	BZ	1	82.71	3.88	0.05
	EG	2	78.96	2.13	0.34
	Step 2 conclusion: drop term EG				
3	<none>		78.96		
	BZ	1	81.12	4.16	0.04
	Step 3 conclusion: MAM = IBP ~ BZ + ε				

CAPÍTULO 4

The role of vegetation structural complexity in tropical passerine cheating behavior

Este manuscrito será submetido ao periódico *Behavioral Ecology and Sociobiology* após a apreciação pela banca examinadora. Portanto, este segue o padrão de referência bibliográfica do periódico.

Abstract

Despite the large body of studies reporting cheating behaviors in breeding, such as extra pair paternity (EPP) and intraspecific brood parasitism (IBP), few authors have addressed hypotheses about how habitat can influence these behaviors. Several studies suggest that vegetation structural complexity can increase levels of EPP and IBP. Here we investigate if a descriptor of habitat complexity (shadow intensity) can predict the occurrence of EPP and IBP, in blue-black grassquits *Volatinia jacarina*. Blue-black grassquits are sexually dimorphic birds, abundant in the Neotropics, and which exhibit high levels of cheating behaviour (EPP). Across the breeding season, males perform sexual displays that have a high dependency upon habitat structure. During four breeding seasons, we searched for grassquit nests in a free-living population in central Brazil, and collected data on vegetation around nests. We found that habitat shadowing increases the occurrence of IBP, but found no association between degree of shadowing and EPP. We suggest, that despite the cost of care an IBP, it can be counterbalanced if shadowed improve nest camouflage and decrease predation rate. Our findings support the evidences that habitat imposes limitations for cheating behavior. We highlight that the shadow is an extreme importance parameter of breeding success of the blue-black grassquit.

Key words: breeding; cuckoldry; egg dump; infidelity; fidelity; monogamy; paternity; sexual selection

Introduction

In the last few decades, the use of molecular genetic analyses has shown that cheating behaviors in breeding, such as extra pair paternity (EPP) and intraspecific brood parasitism (IBP), are more common among birds than previously thought (Lack 1968). Several hypotheses have been proposed to explain the general patterns of EPP (Westneat et al. 1990; Griffith et al. 2002; Neudorf 2004) and IBP (Yom-Tov 1980; Rothstein 1990). Surprisingly, few authors address questions of whether habitat characteristics can influence cheating behaviors. One habitat parameter that can potentially limit cheating behavior is vegetation complexity, which is directed linked to mate-guarding and intrusion detection (Mays and Ritchison 2004).

Vegetation complexity can be defined as plant architecture or connectivity of plant parts (Randlkofer et al. 2010). This characteristic can be linked to the level of concealment for birds, since vegetation structure acts as a filter that limits light and sound propagation (Ingebjørg et al. 2005). High vegetation complexity can be linked to cheating behaviors because it conceals intruders when performing extra pair copulations and egg dumping. Higher vegetation density also reduces an individual´s ability of maintaining close proximity to its mate (Mays and Ritchison 2004) and consequently reduces mating-guarding efficiency (Sherman and Morton 1988; Westneat and Sherman 1997; Westneat and Stewart 2003). Additionally, low luminosity generated by dense vegetation may reduce the accuracy in recognizing parasitic eggs (Rothstein 1975; Honza et al. 2014), which is a key element for parasitic egg ejection (Soler et al. 2014).

These observations suggest that habitat complexity may predict cheating behavior (Mee et al. 2004; Muck et al. 2009; Biagolini-Jr et al. 2017).

A previous study based upon literature reviews and that applied a phylogenetic comparative approach showed a marginally significance effect of habitat structural complexity on broad patterns of EPP (Biagolini-Jr et al. 2017). However, the study lacked an accurate measurement of habitat complexity. Here, using the Neotropical blue-black grassquit *Volatinia jacarina* as our model, we test whether vegetation vertical structural complexity is associated with the occurrence of EPP and IBP. Previous studies show that blue-black grassquit breeding depends on vegetation complexity in various contexts. During the breeding season, males perform multimodal displays (Carvalho et al. 2007), which are used by females in mate choice (Manica et al. 2016) and are an honest signal of territory quality (Manica et al. 2014a). Males use exposed perches from which to initiate their leaping displays (Manica et al. 2016). The display imposes a trade-off between attracting mates and predators (Dias et al. 2010; de Moraes et al. 2019). Males increase displays rates when exposed to a high incidence of direct sunlight (Sicsú et al. 2013) and additionally, increase leap duration when the lower vegetation stratum offers shadowing (Chapter 1).

We hypothesized that cheating behavior is related to vegetation vertical structural complexity. We based this hypothesis upon the assumption that denser vegetation confers a higher degree of concealment that would allow a higher incidence of both EPCs and parasitic egg laying. To test this, we used a free-living population of blue-black grassquits to examine if the occurrence of EPP and IBP is associated with vegetation structural complexity parameters, using data of shadow around nests as a proxy of vegetation complexity.

Methods

Study site and species

We carried out this study within the University of Brasilia campus, in Brasilia, Brazil (15°45'S; 47°52'W; altitude ca 1000m), from December to March across four breeding seasons (2015-2019). The study area´s vegetation is classified as Cerrado *sensu stricto* (tropical savanna) with a high diversity of woody trees (Assunção and Felfili 2004) and grasses (Aguilar et al. 2008).

The blue-black grassquit is an abundant species found in Neotropical grasslands. In central Brazil, breeding activity occurs during the rainy season, from December to March, which is associated with a high grass seed abundance (Carvalho et al. 2007). Nests are spatially aggregated (Dias et al. 2009), and nest site selection is based on vegetation structure, but not on species composition (Aguilar et al. 2008). Both sexes build the nest and provide parental care, but cheating behavior is extremely common (Carvalho et al. 2006; Manica et al. 2016). Predation is considered the main factor leading to lack of breeding success (Almeida and Macedo 2001; Carvalho et al. 2007; Aguilar et al. 2008; Dias et al. 2010).

Data collection

Nests were found by taking slow walks within the study area. In the first two breeding seasons, we collected the eggs after a minimum of three days of incubation, and from these we obtained tissue samples from embryos and extracted DNA for genetic analyses. Nest desertion was assumed when parents no longer attended the nest for at least 10 days. Deserted eggs were also collected to DNA sample. For nests found in the nestling period, and for nests in the last two reproductive seasons, we collected blood samples by brachial venipuncture when nestlings were at least 3 days old.

When nests were no longer active, we collected vegetation data in a radius of 3 m around the nests. We did not demarcate parental territories, but assume that the sampled area had a high probability of falling within territories (Aguilar et al. 2008). We estimated vegetation complexity by estimating two habitat parameters: shadow strength and vegetation structural aggregation. Shadow strength is a static measure of the proportion of sunlight captured by the vegetation. As vegetation growth is slow, shadow strength within each territory can be considered constant across the breeding season. Shadow strength was estimated as the slope of a linear regression of light-meter measures (Extech 401025) and height at which each measure was taken: 200, 180, 150, 120, 50, 40, 30, 20 and 10 cm above ground. Although maximum vegetation height was not constant across the study site, most of the vegetation was below 200 cm. Shadow strength data were collected at 12 points around nests, located at 1, 2 and 3 m from each nest, in the four cardinal directions. All light measures were taken in approximately 20-30 seconds, and values were voice recorded in smartphones and followed up with computer transcriptions.

Genetic analyses

We evaluated the occurrence of EPP and IBP by using molecular markers to estimate kinship among offspring within each nest. We assumed that broods containing only full siblings (FS) resulted from monogamous mating of the social parents. We considered that EPP and IBP had occurred in broods containing half siblings (HS) or genetically unrelated (UR) offspring, respectively. Our method does not allow us to estimate cheating behavior in nests with only one nestling, or the simultaneous occurrence of EPP and IBP in nests with two nestlings.

We performed amplifications of 15 microsatellites previously tested by Manica et al. (2016; see Table 1). Using the software Cervus 3.0 (Kalinowski et al. 2007), we assessed the quality of the genotypic dataset by calculating allelic diversity, observed (HO) and expected heterozygosity (HE), probability of heterozygosity deficit, probability of Hardy-Weinberg equilibrium, exclusion probability for the first and second candidate parent, probability of identity, and probability that the set of loci could not exclude an unrelated pair. Detailed information on observed allelic diversity is presented in the supplementary material.

Nestling kinship was estimated by a combination of methods. First, we identified the more likely relationship between nestlings using the ML-Relate software (Kalinowski et al. 2006). Secondly, we confirmed the relationship by testing the hypotheses of FS or HS relationships versus a null hypothesis of UR in the Kingroup software (Konovalov et al. 2004). These two methods agreed in the identification of 96% (308 of 321) of relationships. For the remaining comparisons (n=13), we calculated the triadic likelihood relatedness estimator (Wang 2007) index with a 95% confidence using the Related R Package (Pew et al. 2015). We improved the accuracy of the index by estimating allelic frequencies by sampling one individual from each nest, and taking the average of the observed frequencies in 1000 iterations. For all relationships, triadic index indicated FS status, because the index estimator range contained 0.5 (expected for FS) and excluded values below 0.25 (expected for HS or U). When a case of cheating behavior was detected in nests with two offspring, we removed it from the estimation of the other cheating behavior, since such nests could not exhibit both EPP and IBP simultaneously.

Statistical analyses

To test if the occurrence of EPP and IBP could be influenced by shadow intensity, we fitted Generalized Linear Model (GLM), with a binomial error structure, using the occurrence of EPP (model 1) and IBP (model 2). We included brood size as a cofactor, since larger broods are more likely to exhibit EPP or IBP. Model selection was performed by backward stepwise model selection, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model (MAM). We assumed no evidence to support the tested hypothesis when only brood size or none of the variables (null model) was present in the MAM. Backward stepwise model selection is presented in the supplementary material. We checked model validation, which included homogeneity, residuals normality, and independence of each explanatory variable by graphic inspection (Zuur et al. 2009).

Results

Across four breeding seasons, we found 258 grassquit nests, containing 592 eggs/nestlings. We successfully collected DNA samples from 257 eggs / nestlings from 115 nests. Among sampled nests brood sizes were: 1 (n=11), 2 (n=69), 3 (n=34) or 6 (n=1). After excluding data from nests that had only one nestling, we detected EPP in 19.8% of nests (19 of 96) and IBP in 12.9% of nests (12 of 93). The mathematical modeling indicates that the occurrence of EPP is not influenced by the degree of shadow (model 1). IBP, however, is more often observed in territories with greater shadowing ($\beta = 0.4835$, $\chi^2 = 4.54$, df =1, p=0.03 – model 2) (Table 1).

Discussion

There is a growing number of reports linking avian breeding behavior to habitat characteristics, such as food (Humbird and Neudorf 2008; Lessard et al. 2014; Kaiser et al. 2017), light (Kempenaers et al. 2010; Da Silva et al. 2014) and sound landscape (Fuller et al. 2007; Francis et al. 2009; Shannon et al. 2016). In this study, we tested if vegetation structural complexity can predict the occurrence of EPP and IBP rates, using a tropical bird, the blue-black grassquit, as a model. The blue-black grassquit is an ideal model to test these ideas because the species is highly dependent upon vegetation complexity in some aspects of its breeding. We found an association between the occurrence of IBP and habitat shadowing, but none relative to vegetation aggregation. EPP occurrence was not associated with either shadowing or vegetation aggregation.

Our results show that grassquits, when nesting in more highly shadowed territories, may have lower fitness due to a higher chance of IBP. Our explanation for these results is that shadowed territories provide concealment for egg dumping females and also reduces the ability of hosts to recognize parasitic eggs (Rothstein 1975; Honza et al. 2014). One of our recent studies showed that male display leap duration increases with shadow intensity (Chapter 1). This suggests that high-quality males, capable of conducting displays at higher intensities, are more likely to occupy such territories. In that study, we argued that higher quality males may occupy more shadowed territories because these might provide greater possibilities of males hiding in the shadows when they are not actively displaying, and would additionally also provide more safety for nests and females. However, if vegetation density, although providing protection against predators, may have the negative consequence of providing better cover also for potential nest parasites. Furthermore, nest parasites could use displaying males as cues for finding nests, similar to the nest-finding behavior exhibited by nest predators (Dias et al. 2010).

A previous experimental study in the same study area, which mimicked parental behavior of ejecting a single parasitic egg from the nest, showed an increased predation rate of the second egg in the clutch (Biagolini-Jr and dos Santos 2018). For blue-black grassquits, which lose approximately 70% of their nests to predation (Aguilar et al. 2008), the cost of caring for a parasitic offspring may be lower than the cost of parasitic egg ejection (Soler et al. 2014). Vegetation species composition does not influence predation rate (Aguilar et al. 2008), but it is possible that a shadowed habitat can improve nest camouflage and decrease predation rate.

Understanding the role of vegetation structure in avian life history remains an important challenge for future studies. Despite the numerous publications examining the associations between vegetation structure and bird breeding behavior (reviewed in Biagolini-Jr et al. 2017; Menezes and Santos 2020), the definition of global patterns is still limited by the lack of data from tropical habitats (Macedo et al. 2008; Xiao et al. 2017). Grassquits are an exception in this regard as there is a significant body of work showing multiple associations between grassquit breeding performance and habitat conditions. For instance, vegetation height and ground coverage are important predictors for nest placement (Aguilar et al. 2008). Moreover, it has been suggested that the presence of tall trees inside territories can increase the range of the display´s vocal component (Wilczynski et al. 2010; Manica et al. 2014b). Finally, displays executed from high perches may be important to increase visibility of male plumage iridescence through exposure to direct sunlight (Sicsú et al. 2013). Thus, vegetation complexity may be extremely important for the survival and breeding success of blue-black grassquits. Here, we have explored another important link between habitat and breeding behavior and shown that habitat complexity influences the occurrence of IBP. In conclusion, IBP is more frequently observed in shadowed territories, which may provide cover for brood parasites and reduce host ability of recognizing parasitic eggs. We speculate that nest parasites may use the exuberant displays of males that typically occupy more shadowed territories as cues for finding nests (Dias et al. 2010). We suggest that future studies should also evaluate how habitat components can limit grassquit communication, and test if there is an effect of shadowing on predation risk and nestling success.

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Conflict of interest - The authors declare that they have no conflict of interest.

Permits - Methods used in this study are in accordance with ethical standards and Brazilian laws. The relevant licenses for this project are: Bioethics Committee of Universidade de Brasília license no. 66711/2016; Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) license no. 51639; and Centro Nacional de Pesquisas para Conservação das Aves Silvestres (CEMAVE) license no. 4255.

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Table

Table 1. Model II coefficients estimation (β), standard errors (Std. Error), z and p values from term significance test. Intraspecific brood parasitism (IBP) is the response variable, and shadowing intensity is the predictor variable.

Coefficients	β	Std. Error	z	p
Intercept	-2.96	0.641	-4.621	<0.001
Shadowing	0.483	0.225	2.143	0.032

Supplementary material

Table S1. Characteristics of 15 microsatellite loci tested for kinship analyses. Calculation of all parameters performed in Cervus 3.0 (Kalinowski et al. 2007), microssatellite sequency descbed at Manica et al. (2016). Hobs = observed heterozygosity, HExp = expected heterozygosity, NE-1P = Average non-exclusion probability for one candidate parent, NE-2P = Average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex, HW = significance of deviation from Hardy-Weinberg equilibrium. ND = not tested, NS = not significant.

Locus	Number of alleles	HObs	HExp	NE-1P	NE-2P	HW	Null allele frequency
Breading season 2015-2016							
VJE5	20	0.855	0.921	0.29	0.17	ND	0.0341
VJJ13	13	0.892	0.872	0.414	0.26	NS	-0.0189
TG11-011	7	0.675	0.736	0.669	0.492	NS	0.0479
GF11	8	0.59	0.569	0.827	0.679	NS	-0.0212
GF12	19	0.843	0.891	0.356	0.217	NS	0.0274
TG03-098	5	0.602	0.534	0.855	0.727	NS	-0.0787
GF16	4	0.06	0.059	0.998	0.97	ND	-0.0069
TG04-061	4	0.422	0.392	0.923	0.791	ND	-0.0463
TG13-017	11	0.771	0.767	0.607	0.423	NS	-0.0086
GF14	8	0.229	0.246	0.968	0.861	ND	0.068
TG02-088	5	0.578	0.485	0.881	0.764	NS	-0.0991
GF01	8	0.361	0.439	0.896	0.746	NS	0.117
TG03-002	5	0.723	0.681	0.74	0.57	NS	-0.03
TG22-001	6	0.494	0.55	0.845	0.717	NS	0.0477
TG01a48	8	0.831	0.765	0.637	0.459	NS	-0.0465
Breading season 2016-2017							
VJE5	25	0.904	0.929	0.26	0.15	ND	0.0115
VJJ13	16	0.79	0.864	0.426	0.269	NS	0.046
TG11-011	11	0.743	0.715	0.692	0.52	NS	-0.0193
GF11	10	0.575	0.561	0.834	0.699	NS	-0.0182
GF12	19	0.868	0.904	0.324	0.193	NS	0.0204
TG03-098	4	0.635	0.561	0.841	0.71	NS	-0.073
GF16	8	0.138	0.132	0.991	0.93	ND	-0.026
TG04-061	7	0.473	0.429	0.899	0.743	NS	-0.0719
TG13-017	12	0.701	0.769	0.6	0.417	NS	0.0529
GF14	11	0.257	0.244	0.968	0.861	ND	-0.0439

TG02-088	3	0.533	0.514	0.869	0.769	NS	-0.0149
GF01	10	0.371	0.41	0.906	0.753	NS	0.0634
TG03-002	5	0.557	0.593	0.815	0.661	NS	0.0274
TG22-001	6	0.479	0.494	0.875	0.752	NS	0.02
TG01a48	8	0.82	0.754	0.636	0.454	NS	-0.0447
Breading season 2017-2018							
VJE5	26	0.901	0.924	0.278	0.162	ND	0.0113
VJJ13	15	0.822	0.877	0.404	0.252	ND	0.0328
TG11-011	6	0.703	0.703	0.718	0.55	NS	0.0012
GF11	5	0.495	0.468	0.889	0.759	NS	-0.0433
GF12	20	0.871	0.891	0.354	0.215	NS	0.0107
TG03-098	4	0.614	0.541	0.852	0.735	NS	-0.078
GF16	6	0.149	0.142	0.99	0.925	ND	-0.0291
TG04-061	4	0.436	0.421	0.907	0.761	NS	-0.0153
TG13-017	10	0.733	0.73	0.664	0.482	NS	-0.0048
GF14	8	0.228	0.23	0.972	0.873	ND	0.0162
TG02-088	6	0.495	0.485	0.881	0.753	NS	-0.0235
GF01	7	0.426	0.444	0.895	0.748	NS	0.0615
TG03-002	5	0.584	0.563	0.837	0.688	NS	-0.0137
TG22-001	6	0.535	0.564	0.834	0.7	NS	0.0115
TG01a48	8	0.733	0.754	0.642	0.463	NS	0.0059
Breading season 2018-2019							
VJE5	27	1	0.953	0.192	0.106	ND	-0.0272
VJJ13	16	0.933	0.885	0.386	0.238	ND	-0.0317
TG11-011	9	0.678	0.734	0.673	0.498	NS	0.0429
GF11	6	0.567	0.527	0.861	0.757	NS	-0.039
GF12	18	0.9	0.912	0.309	0.183	ND	0.0047
TG03-098	4	0.533	0.605	0.816	0.668	NS	0.0399
GF16	6	0.2	0.207	0.978	0.889	ND	-0.0018
TG04-061	5	0.233	0.298	0.954	0.836	ND	0.1414
TG13-017	8	0.6	0.742	0.647	0.463	NS	0.1097
GF14	7	0.244	0.246	0.968	0.865	ND	0.014
TG02-088	5	0.489	0.456	0.896	0.786	NS	-0.0508
GF01	9	0.344	0.397	0.915	0.771	ND	0.0873
TG03-002	5	0.644	0.591	0.814	0.655	NS	-0.0384
TG22-001	4	0.489	0.535	0.856	0.74	NS	0.0413
TG01a48	8	0.789	0.737	0.658	0.477	NS	-0.0449

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Table S2. Backward stepwise model selection of Model I, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: Extra Pair Paternity (EPP); predictor variables are: Shadow Index (SI), and Brood Size (BS).

Step	Term	Df	AIC	χ2	p
1	<none>		97.368		
	SI : BS	1	98.523	3.1545	0.07572
	Step 1 conclusion: drop term SI : BS				
2	<none>		98.523		
	SI	1	97.582	1.0589	0.3035
	BS	1	98.475	1.9519	0.1624
	Step 2 conclusion: drop term SI				
3	<none>		97.582		
	BS	1	97.52	1.9386	0.1638
	Step 3 conclusion: MAM = EPP ~ 1 + ε				

Table S3. Backward stepwise model selection of Model II, using likelihood ratio tests criteria to detect which terms should be dropped to reach the minimal adequate model. Response variable: Intraspecific Brood Parasitism (IBP); predictor variables are: Shadow Index (SI), and Brood Size (BS).

Step	Term	Df	AIC	χ2	p
1	<none>		70.401		
	SI : BS	1	71.889	3.4873	0.06184
	Step 1 conclusion: drop term SI : BS				
2	<none>		71.889		
	SI	1	74.549	4.6604	0.03087
	BS	1	70.986	1.0973	0.29486
	Step 2 conclusion: drop term BS				
3	<none>		70.986		
	SI	1	73.525	4.5389	0.03313
	Step 3 conclusion: MAM = IBP ~ SI + ε				