Renata Calixto Campos

INFLUÊNCIA DO CULTIVO DE MILHO TRANSGÊNICO EM ORGANISMOS NÃO-ALVO (COLEOPTERA: SCARABAEINAE) E DA INGESTÃO INDIRETA ATRAVÉS DA CADEIA TRÓFICA

Tese submetida ao Programa de Pós-Graduação em Ecologia da Universidade Federal de Santa Catarina para a obtenção do Grau de Doutor em Ecologia.

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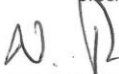"Influência do cultivo de milho transgênico em organismos não-alvo (Coleoptera: Scarabaeinae) e da ingestão indireta através da cadeia trófica"

Por

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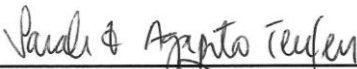
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Florianópolis, 14 de Julho de 2016.

Dedico à minha família

"Absence of evidence is not evidence of absence"
-- Carl Sagan, Astrônomo

AGRADECIMENTOS

RESUMO

O uso de plantas transgênicas ou plantas geneticamente modificadas (GM) que expressam genes com atividade inseticida representam uma alternativa para controlar insetos pragas. As plantas transgênicas resistentes a insetos expressam genes derivados a partir da bactéria *Bacillus thuringiensis* (Bt). Durante a esporulação essa bactéria produz proteínas cristais. A atividade entomopatogênica desse microrganismo deve-se à presença de um núcleo inseticida dessa proteína em forma de cristal. Após a ingestão por insetos suscetíveis, esses cristais são dissolvidos no intestino médio do inseto em pH levemente ácido (no caso de coleópteros) ou alcalino (lepidópteros e dípteros) e o núcleo ativado. Após a liberação do núcleo ativo da proteína este se liga a receptores específicos do epitélio, promovendo a ruptura osmótica das células epiteliais do tubo digestivo, determinando a morte dos insetos. Quando as plantas transgênicas foram desenvolvidas, os cientistas afirmavam que as toxinas fossem altamente específicas e não afetavam organismos não-alvo, no entanto, atualmente, há ainda incertezas sobre o modo de ação de toxinas Bt. No estado de Santa Catarina, no sul do Brasil, 50.000 hectares estão ocupados por cultivos de milho, o principal produto agrícola do estado, com cerca de 90% destes cultivos plantados com variedades transgênicas, seguindo um tipo característico de manejo, já que o fabricante da tecnologia coloca à disposição do agricultor um "pacote tecnológico", com produtos e práticas que orientam este tipo de cultura. Efeitos negativos de cultivos transgênicos na fauna associada ao longo da cadeia trófica são pouco conhecidos e o uso de insetos indicadores pode servir como uma ferramenta para avaliar os efeitos em cascata dos transgênicos sobre a vida silvestre. Os escarabeíneos (Coleoptera: Scarabaeinae), um táxon com reconhecida importância para a manutenção dos processos ecológicos por participar da decomposição da matéria orgânica, vêm sendo utilizados com sucesso como indicadores ecológicos. Assim, a pesquisa teve como principal objetivo avaliar se existe influência dos transgênicos, através da cadeia trófica, na diversidade, no comportamento e na fisiologia dos escarabeíneos. A tese está dividida em quatro capítulos, dois capítulos realizados na região de Campos Novos, sul do Brasil, e dois em laboratório. O primeiro capítulo relata os possíveis impactos dos cultivos de milho sobre as comunidades de escarabeíneos em fragmentos florestais de Mata Atlântica rodeados por plantações de milho transgênico ou convencional, avaliando o tipo de milho, técnicas de manejo, características vegetacionais, presença de

mamíferos e distância espacial. Em 40 fragmentos de Mata Atlântica, sendo 20 circundados por milho convencional e 20 por milho transgênico, através de um protocolo de coleta com 10 armadilhas por fragmento, iscadas com fezes e carne, foi coletado um total de 3454 indivíduos de 44 espécies: 2312 indivíduos de 42 espécies em meio a milho convencional e 1142 indivíduos de 38 espécies em meio a milho transgênico. Diferenças nas comunidades de escarabeíneos foram encontradas e como esperado para áreas fragmentadas, uma maior riqueza de espécies foi observada em fragmentos maiores. A estruturação da comunidade de escarabaeíneos foi explicada pelas características da vegetação, pelo tamanho do fragmento, pela distância espacial e também pelo tipo de cultivo associado a suas técnicas de manejo. Assim, no sul do Brasil, o uso de milho transgênico pode estar acelerando a perda de diversidade e consequentemente os serviços ecossistêmicos providos pelos escarabeíneos podem estar sendo perdidos. O segundo capítulo teve como objetivo avaliar se existe preferência alimentar pela fauna nativa entre milho convencional ou milho transgênico, revelando se os animais conseguem diferenciá-los. Para tanto foram utilizadas armadilhas fotográficas em dez fragmentos florestais de Mata Atlântica, onde foram oferecidas quatro espigas de milho, duas convencionais e duas transgênicas. Dez mamíferos nativos e sete aves foram fotografadas consumindo milho e não houve preferência de consumo por milho convencional ou transgênico. Nesse cenário, se a alimentação transgênica apresentar algum efeito tardio nos animais que se alimentam desse tipo de milho, animais nativos também irão sofrer tais efeitos já que não conseguem diferenciá-lo. Além disso, se o transgênico passa através das fezes destes animais ao longo da cadeia trófica, a fauna coprófaga poderá sofrer tais efeitos. O terceiro capítulo teve como objetivo testar se a alimentação indireta com milho transgênico pode causar perda de "*fitness*" nos besouros escarabeíneos. Para tanto, escarabeíneos foram alimentados em laboratório com fezes de porco seguindo dois tratamentos: um grupo foi alimentado com fezes de cinco porcos que tinham consumido milho transgênico e outro grupo com fezes de cinco porcos que tinham consumido milho convencional. Em um experimento de olfatometria, os indivíduos de *Canthon rutilans* que foram alimentados com transgênico levaram mais tempo para chegar ao recurso; em um experimento de incorporação de matéria orgânica no solo, a quantidade de fezes enterrada pelos indivíduos de *Coprophanaeus saphirinus* foi maior para aqueles que se alimentaram com convencional; a incorporação de matéria orgânica por casais de *C.*

rutilans, assim como o sucesso reprodutivo, foi similar nos dois tratamentos, embora os que se alimentaram com transgênico produziram mais bolas ninhos. Podemos supor que estas diferenças encontradas no "*fitness*" dos escarabeíneos podem afetar as funções destes nos ambientes naturais, deixando-os menos eficientes na remoção e enterrio das massas fecais. O último capítulo teve como objetivo determinar se a alimentação indireta com milho transgênico pode ter efeito na ecofisiologia dos escarabeíneos, impossibilitando a detecção dos estímulos presentes no ambiente, para isso, avaliamos a resposta antenal, as taxas respiratórias e a atividade da fenoloxidase (PO) de escarabeíneos submetidos aos dois tratamentos alimentares descritos no capítulo três, à base de milho convencional e transgênico. No experimento de eletroantenografia, nenhuma diferença foi detectada nas respostas antenais dos escarabeíneos, entretanto nos experimentos de respirometria e atividade de fenoloxidase foram detectadas evidências de aumento da atividade do sistema imune: aumento da emissão de CO_2 durante a respiração e aumento da atividade de fenoloxidase nos besouros que consumiram uma dieta com transgênico, o que pode ser entendido como uma forma de manter o equilíbrio do sistema imunológico em um ambiente prejudicial, sendo possível que o transgênico esteja atuando como um estressor ambiental para organismos não-alvo (escarabeíneos). A maioria dos estudos publicados sobre os efeitos dos transgênicos em organismos não-alvo não mostrou diferenças com base na mortalidade, no entanto, efeitos mais sutis, tais como mudanças comportamentais ou fisiológicas, não foram estudados em besouros, mas a sua importância é evidente. Assim, os efeitos não-letais demonstrados pelo uso do transgênico nesta pesquisa podem gerar nos besouros escarabeíneos uma menor capacidade competitiva pelos recursos efêmeros e uma maior predação, o que resultaria na diminuição das populações dos mesmos e consequentemente explicaria a perda de diversidade observada nas comunidades em fragmentos de mata nativa, o que pode implicar em prejuízos nos serviços ecossistêmicos providos por estes insetos.

Palavras-chave: escarabeíneos, mamíferos, cultivos, GM, diversidade, preferência alimentar, comportamento, ecofisiologia

ABSTRACT

The use of transgenic plants or genetically modified (GM) plants expressing genes with insecticidal activity represents an alternative to control insect pests. Transgenic plants resistant to insects expressing genes derived from the bacterium *Bacillus thuringiensis* (Bt). During sporulation this bacteria produces crystal proteins. The entomopathogenic activity of the microorganism is due to the presence of an insecticide core of this crystal protein. After ingestion by susceptible insects, these crystals are dissolved in the medium of the insect gut in slightly acidic pH (in the case of Coleoptera) or alkaline (Lepidoptera and Diptera) and the core is activated. Upon release of the active core of this protein, it binds to specific receptors in the epithelia, promoting the osmotic disruption of epithelial cells of the gut, causing the death of the insects. When transgenic plants have been developed, scientists believed that the toxins were highly specific and did not affect non-target organisms, however, currently, are still uncertainties in the mode of action of Bt toxins. In the state of Santa Catarina, in southern Brazil, 50,000 hectares are occupied by maize crops, the state's main agricultural product, about 90% of these crops are planted with transgenic varieties, following a characteristic type of management, the manufacturer of technology offers to the farmer "a technological package" with products and practices that guide this type of culture. Negative effects of GM crops on wildlife associated along the food chain are not well known and the use of insect's indicators can serve as a tool to assess the cascade effects of GM crops on wildlife. The dung beetles (Coleoptera: Scarabaeinae), a taxon of recognized importance to the maintenance of ecological processes to participate in the decomposition of organic matter, has been successfully used as ecological indicators. Thus, the research aimed to assess whether there is influence of GMOs, through the food chain in diversity, behavior and physiology of dung beetles. The thesis was divided into four chapters, two chapters made in the region of Campos Novos, southern Brazil, and two in the laboratory. The first chapter reports the potential impact of maize crops on dung beetles communities in forest fragments of Atlantic Forest surrounded by transgenic or conventional maize plantations, assessing the type of maize, management techniques, vegetation characteristics, presence of mammals and spatial distance. In 40 fragments of Atlantic Forest, 20 surrounded by conventional maize and 20 surrounded by transgenic maize, through a collection protocol with

10 traps per fragment, baited with feces and meat, a total of 3454 individuals of 44 species were collected: 2312 individuals of 42 species among conventional maize and 1142 individuals of 38 species in the midst of transgenic maize. Differences in dung beetles communities were found and as expected for fragmented areas, greater species richness was found in larger fragments. The structure of Scarabaeinae community was explained by the characteristics of the vegetation, the fragment size, the spatial distance and the type of farming associated with their management techniques. Thus, in southern Brazil, the use of transgenic maize may be accelerating the loss of diversity and therefore the ecosystem services provided by dung beetles may have been lost. The second chapter aimed to assess whether there is food preference in native fauna between conventional maize or transgenic maize, revealing if the animals can differentiate them. For this, camera traps were used in ten forest fragments of Atlantic Forest, where they were offered four cobs of maize, two conventional and two transgenic. Ten native mammals and seven birds were photographed consuming maize and no preference of consumption by conventional or transgenic maize was detected. In this scenario, if the GM food present a delayed effect on animals that feed this type of maize, native animals will also suffer such effects, as it cannot differentiate them. Moreover, if the GM passes through the feces of these animals along the food chain, the coprophagous animals can suffer such effects. The third chapter aimed to test if the indirect feeding of transgenic maize can cause loss of "fitness" in dung beetles. To this end, dung beetles were fed in a laboratory with pig feces following two treatments: one group was fed with feces of five pigs who had consumed transgenic maize and another group with feces of five pigs who had consumed conventional maize. In an olfactometry experiment, the *Canthon rutilans* individuals who were fed GM took longer time to get in the resource (feces); in an experiment of incorporation of organic matter in the soil, the amount of feces buried by individuals of *Coprophanaeus saphirinus* was higher in those who were fed feces with conventional maize; the incorporation of organic matter by *C. rutilans* couples was similar in both treatments, as well as the reproductive success, while those fed with GM produced more balls nests. We can assume that these differences in the "fitness" of dung beetles can affect the functions of these in natural environments, making them less efficient in the removal and burial of fecal masses. The last chapter was for determine whether the indirect feeding with transgenic maize may have effect on the ecophysiology of dung beetles, making

them unable to detect the stimuli present in the environment for that, we evaluated the antennal response, respiratory rate and the activity of phenoloxidase (PO) of dung beetles submitted to two feeding treatments described in chapter three, with conventional and transgenic maize. In electroantennography experiment, no difference was detected in the antennal responses of dung beetles, but in the experiments of respirometry and phenoloxidase activity were detected evidence of increased activity of the immune system: increase in emissions of CO_2 during respiration and increased of phenoloxidase activity in dung beetles that consumed a diet with transgenic, which can be understood as a way of maintaining the balance of the immune system in an adverse environment, it is possible that transgenic is acting as an environmental stressor to non-target organisms (Scarabaeinae). Most published studies on effects of GM on non-target organisms showed no differences based on mortality, however, more subtle effects such as behavioral or physiological changes have not been studied in dung beetles, but its importance is evident. Thus, non-lethal effects demonstrated by the use of GM in this research can lead to the dung beetles less competitive by ephemeral resources and increase predation, resulting in decreased of populations and thus explain the loss of diversity observed in dung beetles communities in native forest fragments, which can result in losses in ecosystem services provided by these insects.

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INTRODUÇÃO GERAL

PROBLEMA DE ESTUDO

Organismo geneticamente modificado (OGM) é um organismo cujo material genético foi manipulado através de técnicas de DNA recombinante. Apesar da adoção de OGMs por muitos países, a necessidade de pesquisa em biossegurança continua sendo uma preocupação. Tecnologias e intervenções que envolvem sistemas biológicos complexos implicam em incertezas e potenciais riscos acerca de efeitos inesperados e desconhecidos. Existe carência de informações e de consenso científico e tais lacunas limitam o conhecimento com os quais as agências de regulação podem traçar o acesso aos impactos oriundos destas tecnologias na saúde humana e no ambiente.

O uso de plantas transgênicas ou plantas geneticamente modificadas (GM) que expressam genes com atividade inseticida representam uma alternativa para controlar insetos pragas. A maioria das plantas transgênicas resistentes a insetos expressam genes derivados a partir da bactéria *Bacillus thuringiensis* (Bt). Durante a esporulação essa bactéria produz proteínas cristais. A atividade entomopatogênica desse microrganismo deve-se à presença de um núcleo inseticida dessa proteína. Após a ingestão por insetos suscetíveis, esses cristais são dissolvidos no intestino médio do inseto em pH levemente ácido (no caso de coleópteros) ou alcalino (lepidópteros e dípteros) e o núcleo ativado. Após a liberação do núcleo ativo da proteína este se liga a receptores específicos do epitélio, promovendo a ruptura osmótica das células epiteliais do tubo digestivo, determinando a morte dos insetos. A morte também pode ocorrer em função de uma segunda causa associada à primeira, a multiplicação bacteriana na hemolinfa, determinando um processo septicêmico (GILL et al., 1992; MONNERAT & BRAVO 2000). Os detalhes precisos de como as endotoxinas se ligam às células do intestino para matar insetos são mal compreendidos (FERRE & VAN RIE, 2002).

O uso de transgênicos na agricultura vem aumentando globalmente, com um grande acréscimo ocorrendo no Brasil nos anos 2013/2014 (JAMES, 2013). O milho é o principal produto agrícola no estado de Santa Catarina, usado como alimento para suínocultura e avicultura. Em Santa Catarina, 50.000 hectares são cultivos de milho, com 89,6% destes plantados com variedades transgênicas (CÉLERES, 2014). As dúvidas sobre a possibilidade de plantas transgênicas afetarem

organismos não-alvo têm atraído o interesse de pesquisadores e agências reguladoras. Quando as plantas transgênicas foram desenvolvidas, os cientistas afirmavam que as toxinas eram altamente específicas e não afetavam organismos não-alvo (SCHULER et al., 1998; BETZ et al., 2000). No entanto, o modo de ação de toxinas Bt está sujeito a mais incertezas do que no início dos anos 1990, quando as plantas Bt foram inicialmente desenvolvidas e promovidas (VACHON et al., 2012; VAN FRANKENHUYZEN, 2013).

Os efeitos das plantas geneticamente modificadas em organismos não-alvo são altamente controversos. Vários artigos não reportam efeitos (ver MARVIER et al., 2007; WOLFSBARGER et al., 2008; NARANJO, 2009) enquanto outros descrevem significantes efeitos negativos em invertebrados (OBRYCKI et al., 2001; HARWOOD et al., 2005; ZWAHLEN & ANDOW, 2005; HILBECK & SCHMIDT, 2006; OBRIST et al., 2006; ROSI-MARSHALL et al., 2007; MARVIER et al., 2007; HILBECK et al., 2008; WOLFSBARGER et al., 2008; CHAMBERS et al., 2010; DUAN et al., 2010; THEN, 2010; CAMPOS & HERNÁNDEZ, 2015a, 2015b; HOLDERBAUM et al., 2015). Uma meta-análise com 42 trabalhos de campo concluiu que invertebrados não-alvo são menos abundantes em plantações transgênicas comparados com plantações sem inseticidas (MARVIER et al., 2007).

A grande expansão agrícola ocorrida na América do Sul é facilitada pela melhoria das culturas e práticas de manejo do solo que suportam rendimentos mais elevados (TILMAN et al., 2001; LAURANCE et al., 2014). Paisagens humanas modificadas são compostas por um mosaico de ambientes, com diferentes graus de aptidão para a ocorrência e dispersão de indivíduos de espécies nativas e não-nativas (UMETSU et al., 2008). A capacidade de persistir em paisagens modificadas pelo ser humano, no caso dos animais, está relacionada principalmente à capacidade de atravessar habitats não-florestais (UEZU et al., 2008; BANKS-LEITE et al., 2010; BENCHIMOL & PERES, 2013). Assim, a expansão da fronteira agrícola vem associada a uma perda de biodiversidade e a um aumento da fragmentação das florestas nativas (GALINDO-LEAL & CÂMARA, 2003). Setenta por cento da população brasileira vive em áreas de Mata Atlântica e as atividades agrícolas vem afetando progressivamente esse este bioma (RIBEIRO et al., 2009; TABARELLI et al., 2010).

Os proponentes da tecnologia acreditavam que o uso de plantações transgênicas mitigaria muitos dos efeitos negativos dos inseticidas, mas

os insetos não suscetíveis às toxinas podem se tornar pestes secundárias das plantações e causar um dano significante ao cultivo (HILBECK & SCHMIDT, 2006; CATARINO et al., 2015). Sendo os inseticidas uma solução imediata que se encontra facilmente à disposição dos agricultores, os mesmos fazem uso de inseticidas sobre qualquer dificuldade na plantação, assim, o uso da tecnologia de modificação genética pode não estar cumprindo seus objetivos iniciais (CATARINO et al., 2015). Em relação ao manejo, o uso da tecnologia transgênica no Brasil está vinculado a um tipo característico de gestão do milho. O fabricante da tecnologia coloca à disposição do agricultor um "pacote tecnológico", com produtos e práticas que orientam este tipo de cultura (FUCK & BONACELLI, 2009). Os efeitos negativos de cultivos transgênicos na fauna não-alvo, associada ao longo da cadeia trófica, são pouco conhecidos (OBRYCKI et al., 2001; LOVEI et al., 2009). O uso de escarabeíneos, um táxon que se destaca como indicador ecológico e de reconhecida importância para a manutenção dos processos ecológicos, tanto em ambientes agrícolas como naturais, pode servir como uma ferramenta para avaliar os efeitos em cascata dos transgênicos sobre a vida silvestre.

OBJETO DE ESTUDO

A subfamília Scarabaeinae (Coleoptera: Scarabaeoidea: Scarabaeidae) agrupa besouros detritívoros que se alimentam principalmente de excrementos de mamíferos (espécies coprófagas) e de carcaças de animais mortos (espécies necrófagas) (HALFFTER & MATTHEWS, 1966). Foram descritas cerca de 7.000 espécies em todo o mundo, e no Brasil, 618 espécies, número subestimado devido à falta de revisões de gêneros e de pesquisadores (VAZ-DE-MELLO, 2000).

Estes besouros enterram porções do recurso alimentar em galerias subterrâneas construídas logo abaixo do recurso (espécies tuneleiras ou paracoprídeas) ou distante alguns metros (espécies rodadoras ou telecoprídeas) da fonte, e esse recurso é utilizado de substrato para a postura dos ovos e de alimento para as larvas. Outro grupo, de espécies residentes ou endocoprídeas, se alimenta e nidifica diretamente no interior do alimento (HALFFTER & MATTHEWS, 1966; HALFFTER & EDMONDS, 1982; HANSKI & CAMBEFORT, 1991).

Os escarabeíneos apresentam alta correlação de riqueza com vários outros grupos taxonômicos, especialmente com mamíferos (HALFFTER & FAVILA, 1993; DAVIS et al., 2002; NICHOLS et al., 2009;

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BARLOW et al., 2010), sendo considerados bons indicadores de diversidade (BARLOW et al., 2007; GARDNER et al., 2008a) e indicadores ecológicos em estudos sobre monitoramento das alterações ambientais, pois respondem rapidamente aos efeitos causados pela destruição, fragmentação e isolamento das florestas, com mudanças na composição, riqueza e abundância de suas assembleias (HALFFTER & FAVILA, 1993; FAVILA & HALFFTER, 1997; DAVIS et al., 2001; NICHOLS et al., 2007; GARDNER et al., 2008b; HERNÁNDEZ & VAZ-DE-MELLO, 2009; BARLOW et al., 2010).

Os escarabeíneos promovem a remoção do solo e a incorporação da matéria orgânica no ciclo de nutrientes auxiliando na limpeza do ambiente e na regulação das propriedades físico-químicas do solo (HALFFTER & EDMONDS, 1982; HANSKI & CAMBEFORT, 1991). Além disso, a construção de túneis por alguns destes besouros permite a aeração e hidratação do solo, assim como a incorporação dos nutrientes presentes nas fezes, carcaças de animais e frutos que são enterrados nessas galerias (HALFFTER & MATTHEWS, 1966; HALFFTER & EDMONDS, 1982; HANSKI & CAMBEFORT, 1991, NICHOLS et al., 2007).

Algumas espécies de Scarabaeinae possuem alta especificidade de habitat (HALFFTER, 1991) e desta forma, não conseguem estender suas populações para áreas abertas ou de monoculturas (KLEIN, 1989; GARDNER et al., 2008b; ALMEIDA & LOUZADA, 2009).Tais espécies são fortemente influenciadas pela fragmentação e perda de habitat, podendo ter sua distribuição restrita ou mesmo desaparecer localmente (DAVIS & PHILIPS, 2005; HERNÁNDEZ & VAZ-DE-MELLO, 2009).

HIPÓTESE DE ESTUDO

Entre os organismos não-alvo dos transgênicos, os escarabeíneos são um grupo importante em termos de diversidade, abundância, biomassa e relevância funcional dentro das comunidades dos decompositores de massas fecais (NICHOLS et al., 2008). Efeitos dos transgênicos nos escarabeíneos podem ser consequência da presença de DNA ou proteínas transgênicas nas fezes de mamíferos utilizadas como recurso, uma vez que estudos anteriores demonstraram que o DNA e as proteínas transgênicas (Bt) podem passar em fragmentos biologicamente significativos ou intactas através do trato gastrointestinal de mamíferos ou aves (LUTZ et al., 2005; GUERTLER et al., 2010; PAUL et al.,

2010) e consequentemente gerar um rota de exposição aos organismos detritívoros. É importante ressaltar que diferenças na proporção dos grupos funcionais nas comunidades de escarabeíneos já foram detectadas: aumento na abundância do grupo funcional residente e uma diminuição no grupo funcional dos tuneleiros em fragmentos de Floresta Atlântica cercados por milho transgênico (CAMPOS & HERNÁNDEZ, 2015a). A alimentação indireta com milho transgênico poderia causar perda ou diminuição de "*fitness*" nos besouros escarabeíneos e esses resultados explicariam as diferenças detectadas nas comunidades em fragmentos de floresta Atlântica cercados por milho transgênico (CAMPOS & HERNÁNDEZ, 2015a). Por conseguinte, efeitos não-letais nos escarabeíneos poderiam potencialmente impactar as funções ecológicas fornecidas por eles, como a remoção e enterrio da matéria orgânica (BRAGA et al., 2013).

Como os besouros são intrinsecamente associados aos mamíferos, uma vez que utilizam suas fezes como fonte de alimento (HALFFTER & MATTHEWS, 1966; ESTRADA et al., 1999; DAVIS et al., 2002; ANDRESEN & LAURANCE, 2007; NICHOLS et al., 2009) e estes estão diretamente relacionados ao consumo de milho nas áreas próximas a estes cultivos, o uso de ambos os grupos taxonômicos neste estudo pode auxiliar na detecção de diferenças na estruturação das comunidades relacionados com os efeitos das culturas transgênicas em espécies não-alvo. Alterações na dieta dos mamíferos podem ter consequências que resultam em mudanças na composição e diversidade de escarabeíneos através de efeitos em cascata trófica. Assim, provavelmente existe uma relação entre o milho GM e os escarabeíneos, mediada pelos mamíferos que se alimentam de milho, e esta ligação poderia ser utilizada em um contexto de avaliação de risco (Figura 1).
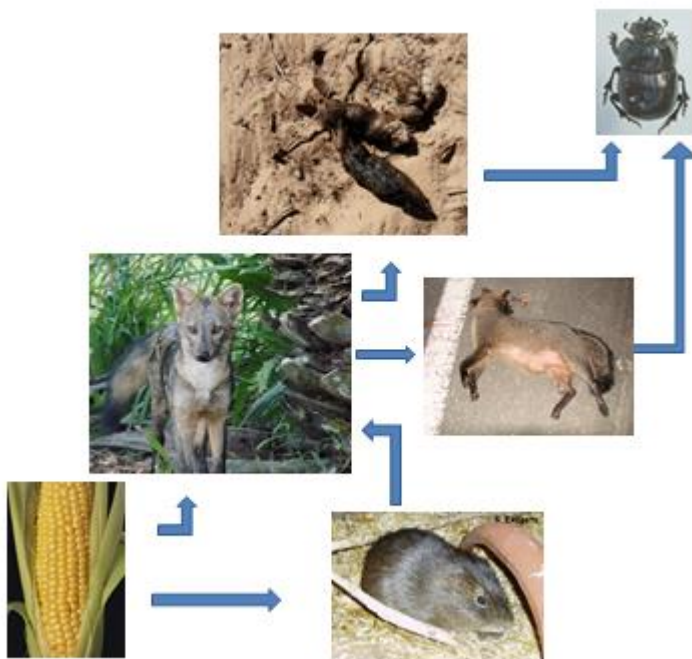
Figura 1: Relação esquemática entre o milho transgênico e besouros detritívoros (escarabeíneos).

Assim, a tese está dividida em quatro capítulos, sendo que cada um será um artigo. Dois capítulos foram realizados na região de Campos Novos, Santa Catarina e dois no Laboratório de Ecologia Terrestre Animal (LECOTA) na UFSC. O primeiro capítulo foi desenvolvido em 40 fragmentos de Mata Atlântica rodeados por plantações de milho: 20 fragmentos rodeados por milho convencional e 20 fragmentos rodeados por milho transgênico, sendo avaliados os possíveis impactos dos cultivos de milho sobre as comunidades de escarabeíneos nos fragmentos florestais. Este artigo baseia-se na hipótese de que o tipo de milho plantado e o tipo de manejo destas áreas influenciam nas comunidades de escarabeíneos presentes nos fragmentos florestais de Mata Atlântica no sul do Brasil.

O segundo capítulo foi desenvolvido na mesma região de estudo, em 10 fragmentos rodeados por milho transgênico, e foi avaliado se existia preferência alimentar pela fauna nativa entre milho convencional ou milho transgênico, revelando se os animais conseguiam diferenciá-los. Este artigo baseia-se na hipótese de que a fauna nativa presente em remanescentes florestais de Mata Atlântica utilizam o milho plantado ao redor dos fragmentos como recurso alimentar, sem escolha pelo milho convencional ou transgênico.

O terceiro e quarto capítulos foram desenvolvidos em laboratório e para serem realizados foram seguidas previamente algumas etapas: plantação de milho transgênico e convencional na Fazenda Experimental da Ressacada, UFSC; fabricação de rações no LAPAD/UFSC para alimentação de porcos; criação de porcos recém-desmamados, com as rações, na Fazenda Experimental da Ressacada, UFSC; coleta e congelamento das fezes dos porcos para posteriormente servirem de alimento aos besouros escarabeíneos.

No terceiro capítulo foram realizados experimentos comportamentais com escarabeíneos alimentados com fezes para avaliar se a alimentação indireta com milho transgênico poderia causar perda ou diminuição de "*fitness*", incluindo detecção de recurso alimentar, manipulação e enterrio do mesmo. Este artigo baseia-se na hipótese de que a proteína transgênica pode ser encontrada nas fezes dos mamíferos que se alimentam com milho transgênico e a presença desta proteína pode causar perda de aptidão nos besouros escarabeíneos que se alimentam dessas fezes.

No quarto capítulo, durante o período de doutorado sanduíche na Universidade de Alicante/Espanha foram realizados experimentos com escarabeíneos alimentados durante um mês para determinar se a alimentação indireta com milho transgênico poderia ter efeito na ecofisiologia dos mesmos, impossibilitando a detecção dos estímulos presentes no ambiente. Este artigo baseia-se na hipótese de que escarabeíneos que se alimentam de fezes de mamíferos, os quais por sua vez se alimentaram de milho transgênico, ficam expostos a proteína Bt derivada do milho transgênico, o que pode provocar alterações ecofisiológicas, impossibilitando a detecção de diversos estímulos químicos presentes no ambiente.

OBJETIVOS

OBJETIVO GERAL
     O objetivo geral deste estudo é avaliar se existe influência dos transgênicos, através da cadeia trófica, na diversidade, no comportamento e na fisiologia dos escarabeíneos.

OBJETIVOS ESPECÍFICOS
Artigo I: **The importance of maize management on dung beetle communities in Atlantic Forest fragments**
Objetivo: Avaliar os possíveis impactos dos cultivos de milho sobre as comunidades de escarabeíneos em fragmentos florestais de Mata Atlântica rodeados por plantações de milho transgênico ou convencional, avaliando o tipo de milho, técnicas de manejo, características vegetacionais, presença de mamíferos e distância espacial.

Artigo II: Do animals have a food preference for conventional or transgenic maize in the Atlantic Forest in Southern Brazil?
Objetivo: Avaliar se existe preferência alimentar pela fauna nativa entre milho convencional ou milho transgênico, revelando se os animais conseguem diferenciá-los.

Artigo III: Indirect exposure to Bt maize through pig feces causes behavioral changes in dung beetles
Objetivo: Testar se a alimentação indireta com milho transgênico pode causar perda de "*fitness*" nos besouros escarabeíneos alimentados com fezes de porcos que foram alimentados com ração à base de milho trangênico.

Artigo IV: Effects of indirect ingestion of GM maize in the ecophysiology of *Canthon rutilans cyanescens* (Coleoptera: Scarabaeidae)
Objetivo: Determinar se a alimentação indireta com milho transgênico pode ter efeito na ecofisiologia dos escarabeíneos, impossibilitando a detecção dos estímulos presentes no ambiente; para isso, avaliamos a resposta antenal, as taxas respiratórias e a atividade da fenoloxidase (PO) de escarabeíneos submetidos aos dois tratamentos alimentares a base de milho convencional ou transgênico.

The importance of maize management on dung beetle communities in Atlantic Forest fragments

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Abstract

Dung beetle community structures changes due to the effects of destruction, fragmentation, isolation and decrease in tropical forest area, and therefore are considered ecological indicators. In order to assess the influence of type of maize cultivated and associated maize management on dung beetle communities in Atlantic Forest fragments surrounded by conventional and transgenic maize were evaluated 40 Atlantic Forest fragments of different sizes, 20 surrounded by GM maize and 20 surrounded by conventional maize, in February 2013 and 2014 in Southern Brazil. After applying a sampling protocol in each fragment (10 pitfall traps baited with human feces or carrion exposed for 48 h), a total of 3454 individuals from 44 species were captured: 1142 individuals from 38 species in GM maize surrounded fragments, and 2312 from 42 species in conventional maize surrounded fragments. Differences in dung beetle communities were found between GM and conventional maize communities. As expected for fragmented areas, the covariance analysis showed a greater species richness in larger fragments under both conditions, however species richness was greater in fragments surrounded by conventional maize. Dung beetle structure in the forest fragments was explained by environmental variables, fragment area, spatial distance and also type of maize (transgenic or conventional) associated with maize management techniques. In Southern Brazil's scenario, the use of GM maize combined with associated agricultural management may be accelerating the loss of diversity in Atlantic Forest areas, and consequently, important ecosystem services provided by dung beetles may be lost.

Introduction

The use of genetically modified (GM) technology in agriculture has increased globally, with the largest increase occurring in Brazil (i.e., an increase of 3.7 million hectares) [1]. The effects of GM plants on non-target organisms are highly controversial. A number of articles have reported no effects (see [2- 4]), while others have described significant negative effects on several invertebrate species [3, 5-13]. A meta-analysis of 42 field experiments concluded that non-target invertebrate groups were less abundant in GM fields compared to insecticide-free fields [2].

The use of GM technology in Brazil is associated with a type of maize management. The manufacturer of GM technology makes available to the farmer, a "technological package" with products and practices that guide this type of culture [14]. Herbicides, for example, are being used in over 70% maize areas in Brazil [15]. Chemical control should be accomplished through the use of herbicides registered and applied in the correct doses. To select an herbicide, the composition of the weeds present, the environmental characteristics in the area to be treated, and the physical and chemical characteristics of the products should be considered [16].

The use of GM crops could mitigate many of the negative effects of insecticides, but insect species that are not susceptible to the expressed toxin can develop into secondary pests and cause significant damage to the crop [8, 17]. Insecticide spraying could become the immediate solution at farmers' disposal, and the sustainable use of this genetic modification technology may be not occurring [17]. The negative effects of GM crops on associated fauna via trophic webs are poorly understood [5, 18]. Currently, the actions of Bt toxins (extracted from *Bacillus thuringiensis*) are subject to more controversy than when Bt plants were first developed [19]. Transgenic DNA and proteins may pass through mammalian or avian gastrointestinal tracts [20-22], as well as through animals that consume them, where transgenic DNA and proteins circulate in the blood and internal organs [23]. The propagation effect of a disturbance at the trophic level to other levels of the food chain may also be occurring. For example, when honeybees were exposed to a high concentration of Cry1Ab protein (GM maize) the effects were not lethal,

but their behavior and learning ability was disrupted [24]. Subtle effects such as aberrations in behavioral or social competence have not been studied to a comparable extent, but these effects may increase or decrease population and community size. The use of some taxon with acknowledged importance in maintaining ecological processes can serve as a tool for finding general patterns related to GM crops cascade effects on wildlife.

A recent study with dung beetles showed changes in functional group dynamics and abundance of some species in communities inhabiting forest fragments surrounded by GM maize [13]. Indirect behavioral effects, for example the search and exploitation of food resources, can generate cascade effects. If a feces provider (mammals) changes their diet, this may have consequences that result in changes to dung beetle communities via trophic cascade effects. Dung beetles (Coleoptera: Scarabaeinae) are extremely important organisms for tropical ecosystem functioning [25] since they promote soil removal and incorporation of organic matter in nutrient cycling, which helps to regulate and improve physical and chemical properties of soil [26-28]. Most species are detritivores, feeding and nesting on feces (coprophagous) or carcasses (necrophagous), both primarily from mammals [29].

Environmental degradation causes changes in dung beetle community structure and composition, resulting in a decrease of species diversity in comparison to preserved areas [30- 34]. The dung beetles rapid response to habitat alterations has led to their recognition as efficient ecological indicators [31, 35-39]. In addition to community-level changes, some species show increased or decreased abundance in areas with particular characteristics caused by environmental change, such as communities found in forest fragments surrounded by GM maize, that show an increase of dweller species and a decrease in tunneler species [13]. Changes in habitat complexity modify not only the insect communities, but also the fauna associated with forests, reducing the richness of some taxonomic groups while increasing others [40]. Furthermore, since dung beetle communities depend on mammal excrements, they may be influenced by changes in mammalian assemblages, which are also affected by landscape alterations [41- 42].

The expansion of the agricultural frontier increases fragmentation and subsequently the loss of biodiversity in the Atlantic Forest [43]. In recent studies was found a positive correlation between dung beetle

richness and mammal richness and the habitat structure influenced both groups [41-42]. Seventy percent of the Brazilian population lives in the Atlantic Forest, one of the most diverse regions in the world, however these human activities have disturbed this ecosystem [44]. More than 80% of Atlantic Forest fragments are smaller than 50 ha and there is a large average distance between fragments (1440 m) [45]. Dung beetle spatial distribution may be related to geographic distance or lack of connectivity caused by fragmentation [46], and due to limitations in dispersal ability [47-49].

This study was based on the hypothesis that dung beetle communities in forest fragments surrounded by genetically modified maize crops (GM) may be exposed to plant materials and toxins derived from transgenic maize via feces or carcasses of maize-consuming animals and exposed to the maize management techniques of these maize crops (GM). The aim of the present study was to reveal the possible impacts of GMs crops associated with maize management techniques evaluating the type of maize crop (conventional or transgenic), maize management of these crops and others important recognized factors (environmental effects, mammalian presence, and spatial distance) to dung beetle communities in Atlantic Forest fragments surrounded by conventional and transgenic maize .

Material and Methods
Study area

The study was conducted in the region of Campos Novos, Santa Catarina state, Southern Brazil (27°23'S, 51°12'W). This region contains several Atlantic Forest fragments, originally Araucaria Forest [50], surrounded primarily by soybean and maize crops. The region has a mild mesothermal climate according to the Köppen classification system with an altitude ranging from 739 to 953 m and distributed rainfall throughout the year, with annual average of 1750 mm approximately [51].

Forty sample areas were established within forest fragments, twenty fragments were surrounded by GM maize crops (ten fragments per year), and twenty fragments were surrounded by conventional maize crops (ten fragments per year) (Fig 1). Farms were chosen with the assistance of the Enterprise for Agricultural Research and Rural Extension of Santa Catarina (Empresa de Pesquisa Agropecuária e Extensão Rural - Epagri/Campos Novos), based on their accessibility

and degree of isolation of forest fragments in relation to the type of maize cultivation. Only forest fragments adjacent to monocultures were chosen.
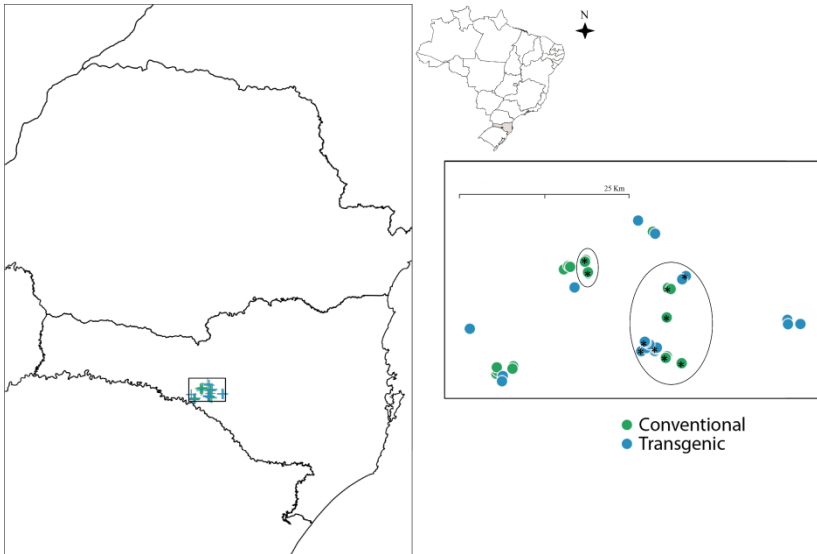
Fig 1: Map of the study region. Location of the 40 forest fragments in the Campos Novos, Santa Catarina state, southern Brazil, near conventional or transgenic maize crops. The twenty fragments sampled in the first year (2013) are circled.

Scarabaeinae sampling

Sampling of copro-necrophagous beetles was performed within the forest at a distance of at least 10 m from the fragment's edge. Each fragment was sampled only once during the study's two years. Sampling was intensive during the summer, in February 2013 and 2014, during the period right before the maize harvest. Pitfall traps were made using plastic containers (30 cm circumference and 20 cm height), buried in the ground, and protected against rain with plastic cap, lastly, a detergent/water mixture and 10 g of bait were added to each trap. The each sampling point consisted of ten traps per fragment, with 400 pitfall traps across the 40 forest fragments sampled. The fragment was used as the sampling unit.The half of traps was baited of human feces and other half with carrion (pork meat) in order to attract the majority of species (i.e., both coprophagous and necrophagous).

After 48 h of exposure, insects captured were fixed in 70% alcohol and taken to the Laboratory of Terrestrial Animal Ecology (LECOTA/UFSC) for identification [52]. Subsequently the insects were deposited in either the Entomological Collection of the Center for Biological Sciences, Federal University of Santa Catarina (UFSC) or the Entomological Collection of Federal University of Mato Grosso (UFMT). Ten individuals per species were weighed (dry weight) using an analytical balance. To find the total biomass of each fragment, the number of individuals was multiplied by the mean biomass per species. The Instituto Chico Mendes de Conservação da Biodiversidade ICMBio/MMA) issued the permits to collect specimens (permit #32333–3 to MIMH). The field study did not involve any endangered or protected species.

Assessment of explanatory variables

Environmental variables – In order to assess the structure of the vegetation in each sampled fragment, an adapted quadrat-section method was used [53]. Using a cross as a reference, four quadrants (northeast, northwest, southeast, southwest) were marked, and in each quadrant the first tree to the center of the cross with diameter at breast height (DBH) greater than 5 cm and the first shrub with a perimeter less than 15 cm and a height greater than 1 m were selected, the following were measured for each tree and shrub: the distances to the center of the cross, the height, and the crown and trunk diameter. Trunk diameter was taken at breast height (1.3 m) for trees and ankle height (DAH = 0.1 m) for shrubs. Furthermore, in each quadrant, height of leaf litter in 1 x 1 m square was measured with a ruler, and percentages of leaf litter layer, green area and exposed soil (no vegetation or leaf litter) were measured by visual estimation using the following classes, 0-5%, 6-25%, 26-50%, 51-75%, 76-95% and 96-100%. Using these same classes, the percentage of canopy cover in the four quadrats was visually estimated, using a square paperboard with a hollow area of 10 x 10 cm, placed at a distance of 40 cm from the observer's eye, at an inclination of 20° in relation to the zenith. For each variable measured was used an average of eight measurements taken between the pitfalls, in two points in the fragment. The area of each fragment was determined using Google Earth Path (1.4.4a), and altitude using a hand-held GPS.

Management of crop fields - Issues regarding crop management were obtained through semi-structured interviews with nine farmers and four

employees, where questions related to agricultural practices used in the properties. The questions were about the maize variety, cattle presence after harvest, use of insecticide, herbicide, and fungicide, and if transgenic crops were ever grown on the site evaluated. All respondents authorized the use of interview data, and interviews lasted on average 15 minutes. Three properties only grew transgenic varieties, eight only grew conventional varieties, and two properties grew conventional and transgenic varieties.

Mammal sampling - Camera traps (BUSHNELL Trophy Cam HD) were used to record mammalian presence inside the forest fragments. Only medium and large mammals were included in the analysis due to the difficulty of identifying little mammal species (rodents) with camera traps. Humans were included because in some fragments the pictures depict hunters, revealing that hunting is present inside the forest. One camera trap was placed in each fragment after dung beetle sampling. Maize from the adjacent crop and meat (to attract predators) were used as bait in front of the cameras. Camera traps were active for a minimum of 40 days and maximum of 60 days. Baits were replaced and batteries checked every 20 days and mammals were identified from the photographs.

Spatial variables - Data from geographic coordinates (Universal Transverse Mercator) obtained at each fragment using a hand-held GPS were used to create spatial variables.

Data analysis

The Jackknife 1, Chao 1 and Chao 2 estimators were used to estimate dung beetle richness in sampled fragments, and sampling sufficiency was calculated using EstimateS v.9 [54]. Data were transformed by square root to reduce the influence of common species and differences in total abundance, and a Bray-Curtis similarity matrix was constructed using communities from different fragments. SIMPER [54] was used to determine the contribution of each species to dung beetle community structure. Analysis of similarities -ANOSIM [55] was used to test differences between dung beetle communities.

The matrices of explanatory data were analyzed and ordinations were performed. A Principal Components analysis (PCA) of environmental variables was calculated using Primer [55] and Principal Coordinates Analysis (PCoA) of management variables was calculated using Hamann similarity in R 3.0.1 [56]. Analysis of similarities (ANOSIM)

[55] was used to test differences between the environmental variables, as well as management variables. The mammal richness matrix was used without transformation. Spatial predictors were created using Principal Coordinates of Neighbour Matrices (PCNM) [57- 58], which is part of a set of spatial eigenfunction analyses called Moran's Eigenvector Maps. The response variables of the dung beetles communities were species richness, abundance and biomass per fragment. The relation of the latter variables to predictor variables (such as vegetation (PCA1), management (PCoA1), mammalian richness, spatial distribution (PCNM1), fragments size and altitude) was initially observed in an exploratory analysis with multiple regressions.

Generalized Linear Mixed Models -GLMMs [59] with a Poisson error distribution [56], were used to test effects of each set of explanatory variables and combined effects of explanatory variables on dung beetle communities in the two types of fragments (conventional and transgenic). In GLMMs, type of maize was considered as a fixed factor. In all analyses performed, the fragment was used as the sampling unit.

Results

Dung beetle communities
A total of 3454 dung beetles belonging to 44 species were collected. *Uroxys* aff. *terminalis*, *Dichotomius* aff. *sericeus* and *Onthophagus* aff. *tristis* were the most abundant species in both fragment types, and together the three species accounted for 60% of abundance in fragments near GM maize, and 48% abundance near conventional maize (S1 Table).

Forty-two species and 2312 individuals were collected in fragments surrounded by conventional maize, and species richness per fragment ranged between two to 21. Thirty-eight species and 1142 individuals were collected in fragments surrounded by GM maize, with six to 25 species per fragment. The number of species observed was at least 80% of the species richness values generated by Chao 1, Chao 2 and Jackknife 1 estimators, demonstrating sampling sufficiency (Table 1).

Table 1: Abundance, observed richness, richness estimators Chao 1, Chao 2 and Jackknife 1, mean biomass per fragment, and total biomass calculated for the communities of Scarabaeinae beetles in fragments adjacent to GM and conventional maize in Campos Novos, Santa Catarina state, Brazil.

Ecological measures of Scarabaeinae community	Fragments adjacent to GM maize	Fragments adjacent to conventional maize
Abundance (N)	1142	2312
Richness (S)	38	42
Variation of richness per fragment	2 to 21	6 to 25
Estimated richness		
Chao1	43.24	43.42
Chao 2	45.12	48.10
Jackknife 1	47.5	51.5
Average biomass per individual	0.086 g	0.130 g
Total biomass	76.16 g	114.71 g

In within the 40 analyzing the dung beetle community similarity Atlantic Forest fragments, significant differences were found between dung beetle communities in fragments near conventional and GM maize (ANOSIM r=0.081, p=0.024). The five species that most contributed to the dissimilarity between fragments types were: *U.* aff. *terminalis* (15.25%), *D.* aff. *sericeus* (8.29%), *O.* aff. *tristis* (6.86%), *C. rutilans cyanescens* (6.10%) and *C.* aff. *trinodosum* (5.43%), since they were most abundant in conventional fragments.

Explanatory variables

Environment – According to the Principal Component Analysis (PCA) of environmental variables, both fragment types are homogeneous, with no separation according to the adjacent crop characteristics, transgenic or conventional. Axis 1 (PCA1) represents the "understory" which explained 24.6% of data variation, and was influenced by shrub diameter, shrub height and tree distance. Axis 2 (PCA2) represents the "forest canopy" which explained 18% of data variation, and was influenced by tree height, shrub diameter and tree crown diameter.

However, according to the variation in environmental variables, the fragments are homogeneous (ANOSIM r=0.12, p=0.006), without separation by type of crop (GM or conventional maize).

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Crop management − A range of management combinations were found, including the use of insecticides in GM crops (S2 Table). The use of insecticide in the region aimed to control mainly the fall armyworm *Spodoptera frugiperda* (Smith, 1797), and corn earworms *Helicoverpa zea* (Boddie, 1850) and *Helicoverpa armigera* (Hübner, 1805).

The insecticide B*t* was the most used in conventional crops (five), and diamide and neonicotinoid in transgenic crops (six). The herbicide atrazine was the most used for weed control in conventional crops (nine) and atrazine (14) followed by glyphosate (11) in transgenic crops. Cattle were released after the harvest in conventional crops (12) and eight transgenic crops (S2 Table).

However, according to the variation in management variables, the fragments are homogeneous (ANOSIM r=0.27, p=0.99), without separation by type of crop (GM or conventional maize).

Mammals − A total of 26 large and medium mammal species were found within the forest fragments, of which 21 are native mammals. The majority of species were found in both fragment types. A total of 19 mammal species were found in fragments surrounded by transgenic maize and 25 mammal species in fragments surrounded by conventional maize (S3 Table). Six different mammal species were 'rare' and had only one or two records each. The availability of mammalian dung was not assessed on these small fragments since most mammals are non-resident and visit the fragments opportunistically. The mammal richness ranged from 1 to 11 species per fragment (S4 Table). In many fragments mammal cubs were detected (i.e., *Procyon cancrivorus*, *Cerdocyon thous*, *Mazama gouazoubira*, *Nasua nasua*), revealing that the period before maize harvest coincides with mammal reproduction. The mammal richness was not correlated to fragment size and was also not correlated to dung beetle species richness and abundance.

Spatial configuration - The total study area including the forest fragments and crops was 790 km^2, with a width of 24 km and length of 47 km. The distance between the fragments ranged from 14 m to 6.5 km and the two fragment types are randomly scattered in the area (Fig 1). PCNM analysis was carried out using a truncated distance matrix and eight statistically significant vectors were selected with the Moran index.

Relationship between dung beetles and environment

In a second evaluation, aiming to examine the set of variables that may have influenced dung beetle community, the first variables of each

test (PCNM´s with spatial distances, PCA with environmental variables, PCoA with management variables) were extracted and multiple regressions were performed. Fragment size, altitude and mammal richness were also included as explanatory variables. Regarding species richness, multiple regressions showed that dung beetle species richness was related to fragment type, conventional or transgenic ($t = 3.17$, $p = 0.003$). Furthermore, dung beetle richness was positively correlated with fragment size ($t = 4.76$, $p = 0.003$), spatial distance (PCNM 1) ($t = 5.48$, $p = 0.004$), and management (PCoA1) ($t = 2.00$, $p = 0.003$), conversely, it was not correlated with mammal richness, environment (PCA1) and altitude. The abundance of dung beetles was also correlated to fragment type, conventional or transgenic ($t = 2.24$, $p = 0.03$), and to spatial distance (PCNM1) ($t = 4.5$, $p = 0.007$), and was not correlated to fragment size, management (PCoA1), environment (PCA1), mammal richness and altitude. The total biomass was correlated to fragment type, conventional or transgenic ($t = -2.55$, $p = 0.015$), it was positively correlated to fragment size ($t = 5.26$, $p = 0.001$), and it was negatively correlated with mammal richness ($t = -2.07$, $p = 0.045$), and it had no correlation with spatial distance (PCNM1), management (PCoA1), environment (PCA1) and altitude (S4 Table).

As predicted for fragmented areas, larger fragments had greater species richness (ANCOVA: $R^2 = 0.43$), and dung beetle species richness was greater in fragments surrounded by conventional maize ($F = 11$, $p = 0.002$) (Fig 2).
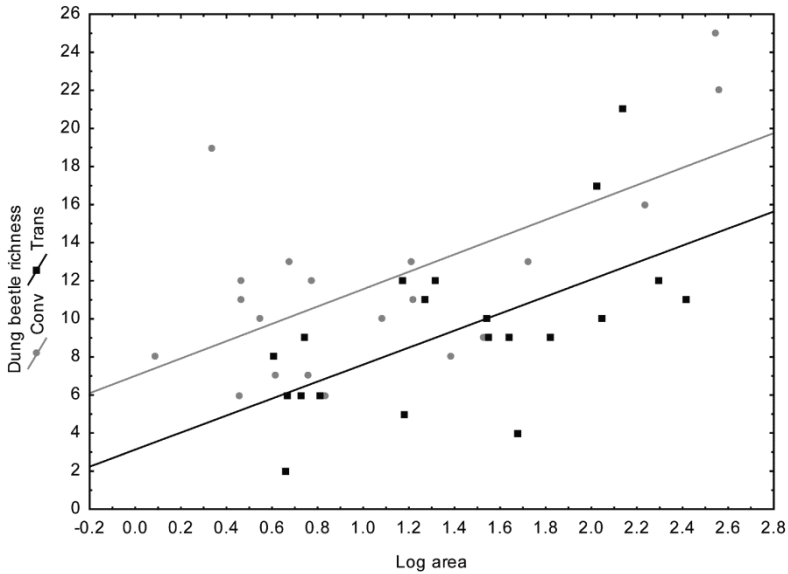
Fig 2: Dung beetle species richness in relation to fragment size (log area) in 40 Atlantic Forest fragments adjacent to transgenic and conventional maize in Campos Novos, Southern Brazil.

Spatial distance (PCNM) was correlated with dung beetle species richness and abundance in both fragment types. Dung beetle species richness and abundance were higher in more distant fragments. However, dung beetle species richness and abundance in fragments surrounded by conventional maize were greater than dung beetle species richness and abundance in fragments surrounded by GM maize. The closest fragments showed similar species richness (Fig 3) and abundance (Fig 4).
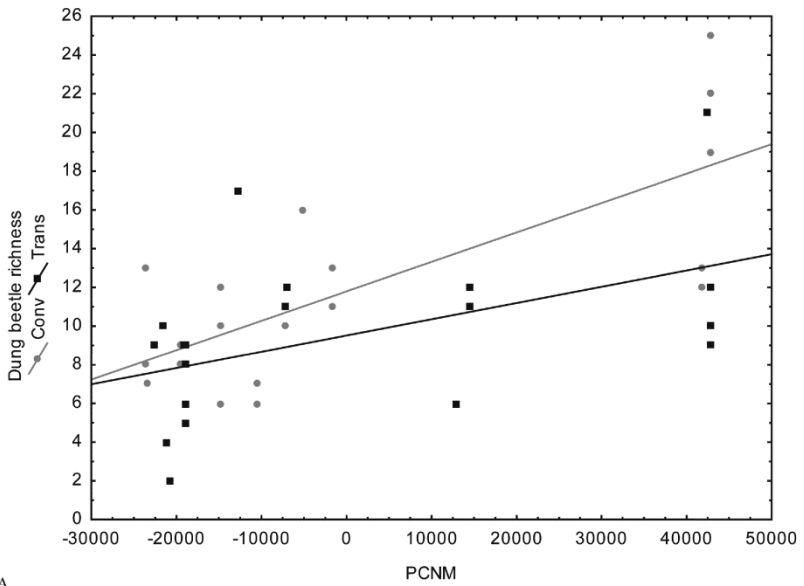
A

Fig 3: Distribution of dung beetle species richness in relation to spatial distance (PCNM) in 40 Atlantic Forest fragments adjacent to transgenic and conventional maize in Campos Novos, Southern Brazil.
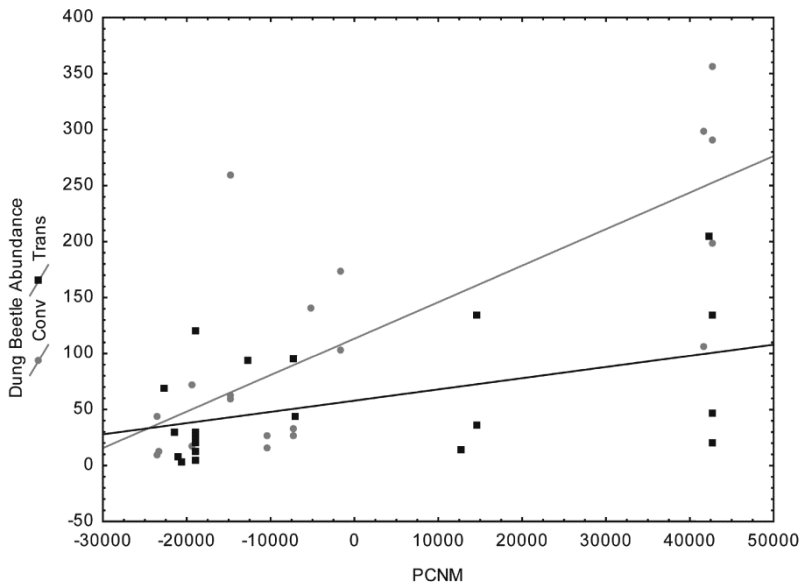
B

Fig 4: Distribution of dung beetle abundance in relation to spatial distance (PCNM) in 40 Atlantic Forest fragments adjacent to transgenic and conventional maize in Campos Novos, Southern Brazil.

When we tested the effects of each set of independent variables: vegetation (PCA), and spatial distance (PCNM) were important explanatory variables for dung beetle communities (Table 2). and the combined effects of variables: type of maize (conventional or transgenic) combined with the PCA (vegetation), type of maize combined with fragment area, type of maize combined with PCNM and type of maize combined with maize management also were important to dung beetle communities (Table 2). The set of variables of management was significant only associated with the factor (conventional or transgenic) (Table 2).

Table 2: GLMM's results of explanatory variables of dung beetle communities in 20 fragments adjacent to transgenic and 20 fragments adjacent to conventional maize, in Campos Novos, Southern Brazil.

Effect	z value	Pr(>|z|)	AIC
Intercept	6.90	<0.001	1650.6
Factor (type of maize)	-1.40	0.160	
PCA (vegetation)	**5.16**	**<0.001**	
Fragment area	-0.65	0.513	
PCNM	8.86	**<0.001**	
Management	1.80	0.070	
PCA *factor	**-2.50**	**0.002**	
Fragment area *factor	**8.95**	**<0.001**	
PCNM *factor	**-1.16**	**<0.001**	
Management *factor	**6.03**	**<0.001**	

Significant effects are in bold.

Discussion

Our results showed that both dung beetle community structure and composition are different in fragments surrounded by GM maize when compared with fragments surrounded by conventional maize, confirming previous findings, where were detected differences in the proportion of functional groups and abundance of some species of dung beetles in Atlantic forest fragments surrounded by GM maize [13]. In addition, dung beetle communities from fragments near GM maize showed lower species richness, total abundance and total biomass. These differences are attributed to maize management techniques and type of maize (conventional or transgenic) surrounding the forest fragment.

Dung beetle species richness at the landscape level reveals a great diversity even in a region with many Atlantic Forest fragments surrounded by a matrix composed of soybean and maize (see [60, 46]). Small forest fragments have been frequently referenced as habitats that are unsuitable for many animals, including large-bodied mammals and associated coprophagous beetles [61]. However, this study found mammal richness to be large in the region, and possibly these mammals use small fragments as stepping-stones or corridors to move to core areas. The majority of mammals registered in this study can disperse for many kilometers and this explains the similarity of mammals within the two fragment types. For example, although the puma (*Puma concolor*)

was recorded in a small fragment (1.2 ha) there are larger fragments in the region. The distribution of dung beetles is strongly influenced by the diversity of mammal excrements [62- 64]. Mammal diversity would explain the high species richness and abundance of dung beetles found in the region, since dung beetle community structure is based on resource availability (bottom up), and the spatial and temporal competition for resources is a strong modifier of dung beetle population dynamics [65].

Fragment size (area) was an important explanatory variable for the dung beetle communities within fragment types, where dung beetle richness and abundance was greater in fragments near conventional maize. It is well known that dung beetles are sensitive to habitat loss and fragmentation and a considerable number of species are forest-dependent [30, 34, 66- 67]. Furthermore, increased dung beetle species richness and abundance was correlated with spatial distance in both fragment types. Spatial limitation of dung beetles may be related to the geographic distance or lack of connectivity caused by fragmentation [46]. The dispersal abilities of different dung beetle species are poorly known, but some research shows that it may vary between 300 and 1500 m depending on the species and landscape [47-49, 68- 69]. It is interesting to note in this study the communities with greater richness and abundance were located in more distant forest fragments in the middle of conventional maize. Since the forest and consequently dung beetles are directly influenced by land use, we suggest that smaller fragments must be managed in order to maintain connected mosaics. Furthermore, the crops surrounding the fragments should be managed to minimize effects on forest fragments and improve connectivity.

Environmental variables influences dung beetle assemblages [34, 60, 70- 71], and the variables related to forest cover (tree and shrub height) shown as important effects on dung beetle variation. These variables are related to factors such as sunlight and humidity, which could affect dung beetle reproduction [72]. Environmental heterogeneity has greater importance at smaller scales [46] and the prevalence of environmental effects indicates species sorting [73]: a metacommunity model where there are strong environmental controls and efficient dispersal, which allows species to track environmental changes [74].

The maize management techniques associated with type of maize cultivated surrounding the forest fragments influences the dung beetles communities present in the fragments. The management effect,

especially the variables 'cattle presence' and 'insecticide use', were important predictive variables for dung beetle community, and 'insecticide' was positively related. However, the study area is predominantly used for agriculture, and insecticide has been applied for many years on the crops, even though within study areas insecticide was not applied during the study's duration, it was applied in previous years. The use of GM maize in these areas was an attempt to decrease application of insecticides (although in seven GM areas insecticide was applied), however the dung beetle community response to this disturbance was worst in terms of dung beetle richness and abundance than in fragments adjacent to conventional maize with insecticide. Thus, the remaining dung beetles species found in the forest fragments in this study are already possibly less affected by this disturbance.

Cattle presence and the indirect use of ivermectin negatively affect the composition and abundance of dung beetles in fragments surrounded by maize. The residuals of ivermectin are released in excrements, which contaminate the environment and can affect dung beetles [75-78]. Some species, unlike the majority, were benefited in fragments where cattle had open access (i.e., *U.* aff. *terminalis*), demonstrating which can be less affected.

Herbicide use was positively related with abundance and negatively with dung beetle biomass. There was an increase in smaller dung beetle species abundance with less biomass. Herbicides are applied in all GM maize crops, as well as most of the conventional maize crops, and herbicide application can cause a decline in the majority of dung beetles and impair reproductive function [79]. Forest-dependent dung beetle species depend in part on their ability to survive in human-modified landscapes [80].

Even in the absence of ecophysiological studies that may determine the effect of GM maize on dung beetle species and, consequently, the effects on dung beetle communities, in Southern Brazil's scenario, where large fields of monocultures threaten biodiversity, the use of GM maize combined with associated agricultural management techniques may be accelerating the dung beetle loss in Atlantic Forest fragments adjacent to cornfields and, subsequently, the loss of ecosystem services provided by dung beetles.

Acknowledgments

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Supporting Information

S1 Table: Scarabaeinae species collected in 40 fragments (February 2013 and 2014) of Atlantic Forest in the region of Campos Novos, Southern Brazil. T: fragments adjacent to GM maize, C: fragments adjacent to conventional maize.

Tribe / Species	T	C	Total
Ateuchini			
Uroxys aff. *terminalis* Waterhouse, 1891	261	912	1173
Uroxys sp.	27	51	78
Coprini			
Canthidium cavifrons Balthasar, 1939	13	12	25
Canthidium aff. *dispar* Harold, 1867	9	8	17
Canthidium moestum Harold, 1867	0	1	1
Canthidium sp	0	7	7
Canthidium aff. *trinodosum* (Boheman, 1858)	81	153	234
Canthidium sp1	1	2	3
Dichotomius assifer Eschscholtz, 1822	1	7	8
Dichotomius bicuspis Germar, 1824	9	19	28
Dichotomius depressicollis (Harold, 1867)	0	1	1
Dichotomius fissus (Harold, 1867)	0	1	1
Dichotomius aff. *punctulatipennis* (Luederwaldt, 1930)	4	5	9
Dichotomius aff. *pygidialis* (Luederwaldt, 1922)	10	2	12
Dichotomius aff. *sericeus* (Harold, 1867)	116	254	370
Dichotomius luctuosus (Harold, 1869)	1	1	2
Homocopris sp.	13	8	21
Ontherus azteca Harold, 1869	4	42	46
Ontherus lobifrons Génier, 1996	0	1	1
Ontherus sulcator (Fabricius, 1775)	1	6	7
Deltochilini			
Canthon angularis Harold, 1868	25	30	55
Canthon auricollis Redtenbacher, 1867	13	3	16
Canthon ibarragrassoi Martinez, 1952	6	16	22
Canthon aff. *laminatus* Balthasar, 1939	2	0	2
Canthon lividus seminitens Harold, 1868	47	45	92
Canthon luctuosus Harold, 1868	17	18	35
Canthon aff. *oliverioi* Pereira and Martínez, 1956	1	4	5
Canthon quadratus Blanchard, 1846	1	3	4
Canthon rutilans cyanescens Harold, 1868	108	145	253

S1 Table (*continuation*): Scarabaeinae species…….

Deltochilum brasiliense (Castelnau, 1840)	48	23	71
Deltochilum cristatum Paulian, 1938	36	63	99
Deltochilum riehli Harold, 1868	5	28	33
Deltochilum rubripenne Gory, 1831	1	0	1
Oniticellini			
Eurysternus aeneus Génier, 2009	0	2	2
Eurysternus caribaeus (Herbst, 1789)	16	2	18
Eurysternus cyanescens Balthasar, 1939	6	2	8
Eurysternus francinae Génier, 2009	13	48	61
Eurysternus navajasi Martinez, 1988	1	2	3
Eurysternus parallelus Castelnau, 1840	35	107	142
Onthophagini			
Onthophagus catharinensis Paulian, 1936	22	3	25
Onthophagus aff. *hirculus* Mannerheim, 1829	4	24	28
Onthophagus aff. *tristis* Harold, 1873	168	230	398
Phanaeini			
Coprophanaeus saphirinus (Sturm, 1826)	14	19	33
Sulcophanaeus menelas (Castelnau, 1840)	2	2	4

S2 Table: Crop management near the 40 forest fragments in Campos Novos, Santa Catarina, Brazil. C: fragments adjacent to conventional maize, T: fragments adjacent to transgenic maize.

	Maize variety	Gene Toxin	Cattle	Inseticide	Herbicide	Fungicide	GM crop ever
C1	Colorado		-	Deltametrina	Atrazine	-	-
C2	Colorado		-	Deltametrina	Atrazine	-	-
C3	Guerra		X	-	Glyfosate-Callisto	-	-
C4	Guerra		X	-	Glyfosate-Callisto	-	-
C5	Guerra		X	-	Glyfosate-Callisto	-	-
C6	Aztec		-	-	-	-	-
C7	Pixirum		X	-	-	-	-
C8	Pixirum		X	-	-	-	-

S2 Table (*continuation*): Crop management near……..

C9	Pixirum		X	-	-	-	-
C10	Pixirum		X	-	-	-	-
C11	AG 8021		X	Diamida	Atrazine-Soberan	-	x
C12	Dow 2B587		-	Bt	Atrazine-Soberan-Simazine	-	x
C13	AS 1570		-	Bt	Atrazine-Soberan-Simazine	-	x
C14	P30F53		X	Bt	Atrazine-Soberan-Simazine	-	x
C15	P2530		-	Bt	Atrazine-Soberan-Simazine	-	x
C16	AG 8021		X	Diamida	Atrazine-Soberan	-	x
C17	AS 1551		-	Bt	Atrazine-Soberan-Simazine	-	x
C18	Pixirum		-	-	0	-	x
C19	Guerra SG 6302		X	-	0	-	-
C20	Guerra SG 6302		X	-	Gramoxone	-	-
T1	P1630H	Cry1F	-	-	Tembotrione-Atrazine	-	x
T2	P1630H	Cry1F	-	-	Tembotrione - Atrazine	-	x
T3	Defender/ Maximus	Cry1Ab	X	-	Zapp + Glyphosate Graminicide	-	x
T4	Defender/ Maximus	Cry1Ab	X	-	Zapp + Glyphosate Graminicide	-	X

T5	Defender/ Maximus	Cry1Ab	X	-	Zapp + Glyphosate Graminicide	-	x
T6	DKB 250Pro	Cry1A.1 05	-	-	Glyphosate Atrazine	Strobilurin - Triazole	x
T7	DKB 250Pro	Cry1A.1 05	-	-	Glyphosate Atrazine	Strobilurin - Triazole	x
T8	DKB 250Pro	Cry1A.1 05	-	-	Glyphosate Atrazine	Strobilurin - Triazole	x
T9	DKB 250Pro	Cry1A.1 05	-	-	Glyphosate Atrazine	Strobilurin - Triazole	x
T10	DKB 250Pro	Cry1A.1 05	-	-	Glyphosate Atrazine	Strobilurin - Triazole	x
T11	AG 8021 YG	Cry1Ab	X	Diamida	Atrazina- Soberan	-	x
T12	AG 8021 YG	Cry1Ab	X	Diamida	Atrazina- Soberan	-	x
T13	Dow 2B587Hx	Cry1F	-	Bt	Atrazina- Soberan- Simazine	-	x
T14	DKB 240Pro2	Cry1A.1 05	-	Imidacloprid	Atrazine- Glyphosate	Strobilurin -Triazole	x
T15	DKB 240Pro2	Cry1A.1 05	-	Imidacloprid	Atrazine- Glyphosate	Strobilurin - Triazole	x
T16	DKB 240Pro2	Cry1A.1 05	-	Imidacloprid	Atrazine- Glyphosate	Strobilurin - Triazole	x
T17	DKB 240 Pro	Cry1A.1 05	X	-	Callisto	-	x
T18	DKB 240Pro	Cry1A.1 05	X	-	Callisto	-	x
T19	DKB 240Pro	Cry1A.1 05	X	-	Callisto	-	x
T20	AG 8021 YG	Cry1Ab	-	Diamida	Atrazine- Soberan	-	x

S3 Table: Mammal species records in 40 Atlantic Forest fragments surrounded by conventional maize (20) or transgenic maize (20) in Campos Novos, Southern Brazil.

Mammal species	Number of fragments with records in 20 fragments surrounded by	
	Conventional maize	Transgenic maize
Mazama gouazoubira (Fischer, 1814)	14	20
Dasypus novemcinctus Linnaeus, 1758	14	14
Cerdocyon thous (Linnaeus, 1766)	11	12
Didelphis albiventris Lund, 1840	12	9
Nasua nasua (Linnaeus, 1766)	8	11
Dasypus septemcinctus Linnaeus, 1758	9	2
Leopardus guttulus (Hensel, 1872)	5	6
Homo sapiens Linnaeus, 1758	4	6
Euphractus sexcinctus (Linnaeus, 1758)	5	4
Bos taurus Linnaeus, 1758	5	4
Canis lupus familiaris Linnaeus, 1758	4	4
Procyon cancrivorus (Cuvier, 1798)	1	4
Dasyprocta azarae Lichtenstein, 1823	2	3
Galictis cuja (Molina, 1782)	2	2
Eira barbara (Linnaeus, 1758)	1	2
Puma yagouaroundi (Saint-Hilaire, 1803)	1	2
Lepus europaeus Pallas, 1778	2	1
Tamandua tetradactyla (Linnaeus, 1758)	1	2
Leopardus wiedii (Schinz, 1821)	2	0
*Cuniculus paca (*Linnaeus, 1766)	0	2

Conepatus chinga (Molina, 1782)	1	0
Sapajus nigritus (Goldfuss, 1809)	1	0
Felis catus Linnaeus, 1758	1	0
Leopardus pardalis (Linnaeus, 1758)	1	0
Puma concolor (Linnaeus, 1771)	1	0
Hidrochaeris hidrochaeris (Linnaeus, 1766)	1	0
Number of species	25	19

S4 Table: Dung beetle community data, mammal richness, and measurements from 40 forest fragments in Campos Novos, Southern Brazil. First principal component (PCA1), first principal coordinates analysis of management (PCoA1) and first principal coordinates of neighbor matrices (PCNM1). C: fragments adjacent to conventional maize. T: fragments adjacent to transgenic maize.

	Year of sampling	Dung beetle richness	Dung beetle abundance	Mammal richness	Fragment area (m²)	Altitude (amsl)	Environment PCA 1	Management PCoA1	Spatial PCNM 1
C1	2013	13	44	3	16.338	932	-0.36	3.20	-23553
C2	2013	8	10	6	24.379	950	-2.07	-3.20	-23539
C3	2013	10	60	8	3.522	826	0.84	-6.60	-14742
C4	2013	12	260	8	5.949	888	0.06	3.26	-14862
C5	2013	6	63	6	6.827	900	0.98	-1.55	-14849
C6	2013	7	13	6	4.136	915	-2.85	-1.96	-23400
C7	2013	8	18	2	1.216	861	-0.08	7.42	-19461
C8	2013	9	72	7	33.681	853	-0.69	9.25	-19447
C9	2013	6	27	3	2.870	847	1.73	7.28	-10405
C10	2013	7	16	4	5.718	833	-0.30	-1.11	-10408
C11	2014	10	33	11	12.006	909	2.56	9.46	-7181
C12	2014	13	107	5	53.216	786	-0.69	-1.31	41764
C13	2014	12	299	6	2.897	751	1.45	1.48	41771
C14	2014	25	357	8	347.615	784	1.16	7.90	42791
C15	2014	19	290	5	2.156	817	1.99	-1.35	42797

S4 Table (continuation): Dung beetle community…..

C16	2014	11	27	4	16.405	910	2.76	8.06	-7179
C17	2014	22	199	5	364.771	773	1.49	-1.43	42798
C18	2014	13	103	5	4.725	793	0.95	3.69	-1671
C19	2014	11	174	5	2.927	768	2.05	-3.72	-1705
C20	2014	16	140	2	172.382	753	0.93	4.89	-5190
T1	2013	10	29	9	111.591	915	0.44	2.14	-21545
T2	2013	9	68	7	35.634	944	-1.62	-2.14	-22637
T3	2013	6	121	8	5.332	878	1.44	9.71	-18952
T4	2013	8	23	5	4.016	893	-2.63	8.71	-18973
T5	2013	9	29	9	65.818	890	-2.97	4.76	-18985
T6	2013	6	12	6	6.517	894	-2.61	-1.52	-18939
T7	2013	5	5	6	15.242	891	3.39	2.65	-18983
T8	2013	9	20	6	43.672	873	0.30	2.65	-19022
T9	2013	2	3	6	4.562	891	-4.09	-2.28	-20665
T10	2013	4	8	4	47.312	896	-2.31	-1.49	-21078
T11	2014	11	96	2	260.237	912	0.18	-1.27	-7192
T12	2014	12	44	6	14.918	853	0.42	-1.27	-7069
T13	2014	21	205	5	18.676	828	-0.15	9.50	42360
T14	2014	6	14	5	136.743	911	1.20	-6.57	12821
T15	2014	11	36	1	4.611	917	-1.52	-5.82	14502
T16	2014	12	134	5	196.514	953	0.30	-6.73	14501
T17	2014	12	20	7	20.764	767	1.25	-4.44	42778
T18	2014	10	47	6	34.505	782	0.41	-6.24	42784
T19	2014	9	134	2	5.554	739	-0.16	-1.25	42763
T20	2014	17	94	5	106.167	872	-3.15	3.00	-12792

Apêndice Capítulo 1: Mamíferos fotografados na região de Campos Novos, Santa Catarina, Brasil, em fragmentos de Mata Atlântica rodeados por cultivos de milho durante os anos 2013 e 2014.

A - Cachorro do mato (*Cerdocyon thous*)

B - Gato maracajá (*Leopardus wiedii*)

C - Irara (*Eira barbaba*)

D - Puma (*Puma concolor*)

E - Veado catingueiro (*Mazama gouazoubira*)

F - Capivara (*Hydrochoerus hydrochaeris*)

G - Tatu galinha (*Dasypus novemcinctus*)

H - Cutia (*Dasyprocta azarae*)

I - Macaco prego (*Sapajus nigritus*)

J - Furão (*Galictis cuja*)

K - Tamaduá mirim (*Tamandua tetradactyla*)

L - Tatu peludo (*Euphractus sexcinctus*)

M – Gambá (*Didelphis albiventris*)

N - Paca (*Cuniculus paca*)

O – Gato do mato pequeno (*Leopardus tigrinus*)

P - Mão-pelada (*Procyon cancrivorus*)

Q - Jaguatirica (*Leopardus pardalis*)

R – Zorrilho (*Conepatus chinga*)

S - Roedor não identificado

T – Lebre (*Lepus europaeus*)
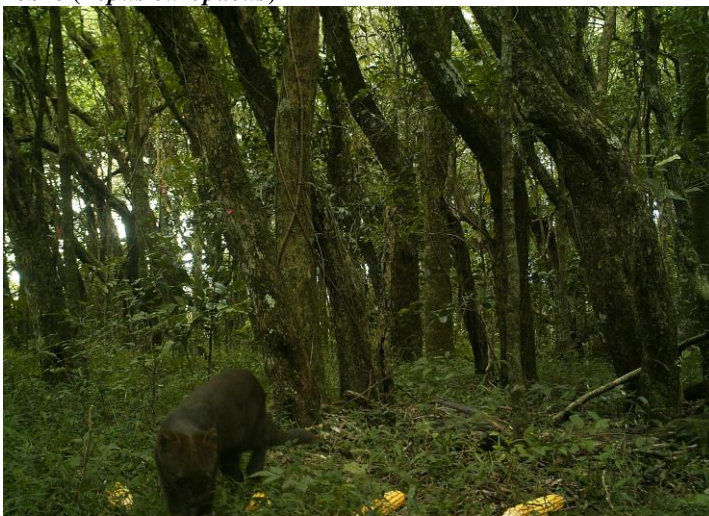
U - Gato mourisco (*Puma yagouaroundi*)

Bushnell Ⓜ C0NV8 70°F21°C ◯   02-28-2013 20:17:30

V - Tatu mulita (*Dasypus septemcinctus*)

Do animals have a food preference for conventional or transgenic maize in the Atlantic Forest in Southern Brazil?

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Abstract

Animals persist in human-modified landscapes due to their ability to cross non-forested habitats like crops. Crops provide resources for native animals, and the search and exploitation of food resources is an important skill for them. In Southern Brazil, a mosaic of plantations increases fragmentation and loss of biodiversity of the Atlantic Forest. The purpose of this study was to assess if there is food preference between transgenic or conventional maize when both are offered to native animals moving across Atlantic Forest fragments surrounded by maize crops. Ten native and one domesticated mammal and seven birds were photographed consuming maize. There was no preference or difference in consumption from conventional to transgenic maize when both types of maize were provided. If transgenic crops have some adverse effect, the wildlife feeding on this type of food will also suffer such effects, since they do not differentiate between them.

Keywords: ecology, behavior, GMO, non-target organisms, wildlife.

Introduction

Human-modified landscapes are composed of a mosaic of environments with different degrees of occurrence and dispersal of individuals from native and non-native species (Umetsu et al., 2008). For animals, the possibilities of persisting in human-modified landscapes are related to their dispersal ability, mainly the ability to cross non-forested habitats, and the use of edge-affected habitats, as well as the absence of hunting or collection (Uezu et al., 2008; Banks-Leite et al., 2010; Benchimol and Peres, 2013). Foraging is the search and exploitation of food resources, an important skill since it affects the animal's fitness, directly influencing the survival and reproduction of

the organism (Danchin et al., 2008). Food preferences appear to change readily and appropriately with changes in the environment (Emlen, 1966).

Improved crops and soil management practices, which support greater harvests, are facilitating the greatest expansion of agriculture in South America (Tilman et al., 2001; Laurance et al., 2014). Thus, the expansion of human-modified landscapes and the consequent reduction of old-growth forests pose a challenge to conservationists devoted to guarantee the persistence of biodiversity (Melo et al., 2013). Moreover, these human activities further disturb this ecosystem and seventy percent of the Brazilian population lives in the Atlantic Forest (Ribeiro et al., 2009; Tabarelli et al., 2010).

Maize is the main agricultural product of Santa Catarina State (Southern Brazil) that supplies feed for pig and poultry farming. More than 50,000 hectares are cultivated with maize in this region, and 89.6% of these are planted with transgenic varieties (CÉLERES, 2014). The use of genetically modified plants (GM) in agriculture has increased globally, with an enormous increase occurring in Brazil in 2013/2014, the 2nd largest adopter of the technology (ISAAA, 2013). The agricultural use of GM technology that expresses genes with insecticidal activity is currently an alternative to control insect pests, however, the management effects of this technology was also observed inside native forest fragments in Southern Brazil (Campos and Hernández, 2015b).

The negative effects of GM crops on wildlife associated with the food chain are poorly known (Obrycki et al., 2001; Lovei et al., 2009). Transgenic DNA and proteins can pass through the gastrointestinal tracts of mammals or birds, intact or in biologically significant fragments (Lutz et al., 2005; Paul et al., 2010; Guertler et al., 2010), and this may affect non-target organisms along the food chain.

MacKenzie and McLean (2002) reviewed 15 feeding studies of dairy cattle, beef cattle, swine and chickens and concluded that the risk to animal health from the use of GM crops as animal feed is negligible. However, effects on native mammals could be occurring, for example, Séralini and collaborators (2014) linked GM maize to the development of tumors and other severe diseases in rats, and effects in pigs were also observed (Carman et al., 2013). Cascade effects may be generated by indirect behavioral effects of transgenic use in agriculture or the search and exploitation of food resources by wildlife, so it is highly important to assess if there are food preferences among native animals for

transgenic or conventional maize inside natural forest patches surrounded by maize crops. Thereby, the purpose of the present study was to evaluate the exploitation of food resources (i.e. maize) to assess the food preference (maize type) among the native fauna, and to reveal if the animals can differentiate between the different types.

Methods
Study area
The trial was carried out in the region of Campos Novos, Santa Catarina State, Southern Brazil (27°23'S, 51º12'W, 947m of altitude). This region contains several Atlantic Forest fragments, originally Araucaria Forest (Leite and Klein, 1990), surrounded mainly by soybean and maize crops. The region has a humid subtropical climate (Cfa), according to the Köppen classification system.

In the summer of 2014, a period before maize harvest, camera traps (BUSHNELL Trophy Cam HD) were used to record animals inside 10 forest fragments. Only fragments surrounded by GM maize crops were chosen in order to not contaminate fragments (transgenic maize in fragments that did not have it), and ten cameras traps were placed, one in each native forest fragment. Four cobs of maize per fragment were used as bait and placed in front of the camera traps to attract animals: two cobs of conventional maize and two of GM maize (all the corn-cobs used in the experiment were from a nearby crop), totalizing 40 cobs. The baits were exposed for 20 days, and after this period of consumption they were measured using the remnants of maize. The cobs were previously identified for further evaluation by marking sticks inserted within: the sticks for transgenic maize cobs were marked with two red lines and conventional maize sticks were marked with only one red line. The four cobs inside the ten forest patches were tied with a nylon thread on nearby trees to prevent its displacement by animals. Consumption by animals in each cob was evaluated using classes ranging from zero to 100%, as follows: 0-5%, 6-25%, 26-50%, 51-75%, 76-95%, 96-100%. Photos were used to identify the animals, and small rodents were identified at family level due to the difficulty of identifying species with camera traps.

To test the difference between the quantities of both maize types consumed in the ten forest fragments, a paired t-test for dependent samples with the average of the two maize cobs in each treatment was

ran, comparing the difference of consumption between samples from the same fragment.

Results

Ten native mammals and one domesticated (cow) in addition to seven birds were photographed consuming maize (Table 1). The gray brocket deer (*Mazama gouazoubira*) was the mammal most photographed consuming maize (present in eight forest fragments), followed by the nine-banded armadillo (*Dasypus novemcinctus*), and the coati (*Nasua nasua*). The plush-crested jay (*Cyanocorax chrysops*) and dusky-legged guan (*Penelope obscura*) were the birds most photographed consuming maize. Within many fragments the young of mammals and birds were detected (i.e., *Cerdocyon thous*, *M. gouazoubira*, *N. nasua*, *Aramides saracura* and *P. obscura*), revealing that the period before maize harvest coincides with animal reproduction. The photographed animals belong to seven foraging categories: insectivorous/omnivorous (27.8%), omnivorous (22.2%), frugivorous/omnivorous (16.6%), frugivorous/herbivorous (11.1%), granivorous (11.1%), frugivorous/granivorous (5.6%), and herbivorous (5.6%) Among mammals most were insectivorous/omnivorous (27%) and among birds most were omnivorous (43%)(Table 1).

The consumption of maize cobs ranged from 0-5% to 96-100%, depending on the forest fragment (Figure 1). In four fragments, the four cobs were completely consumed and in two fragments there was practically no consumption. Regardless of maize type, in each forest patch the cobs were poorly consumed (12 cobs, 0-5%) or totally consumed (17 cobs, 96-100%) (see Figure 1). There was no difference in the exploitation of food resources between conventional or transgenic maize (*t*-value = 0.73, df = 9, p = 0.48) (Table 2).

Table 1: Animal species photographed consuming maize in ten Atlantic Forest fragments surrounded by transgenic maize in Campos Novos, Southern Brazil. Foraging categories: In/Om-insectivorous/omnivorous, Omn-omnivorous, Fr/Om-frugivorous/omnivorous, Fr/He-frugivorous/herbivorous, Gr-granivorous, Fr/Gr-frugivorous/granivorous, He-herbivorous.

Order / Species		Foraging category
Mammals		
Rodentia		
*Cuniculus paca (*Linnaeus, 1766)	Spotted Paca	Fr/He
Dasyprocta azarae Lichtenstein, 1823	Azara's Agouti	Fr/Gr
Cricetidae not identified	Rats	Gr
Didelphimorphia		
Didelphis albiventris Lund, 1840	White-eared Opossum	Fr/Om
Cingulata		
Dasypus novemcinctus Linnaeus, 1758	Nine-banded Armadillo	In/Om
Dasypus septemcinctus Linnaeus, 1758	Brazilian Lesser Long-nosed Armadillo	In/Om
Euphractus sexcinctus (Linnaeus, 1758)	Yellow Armadillo	In/Om
Artiodactyla		
/*Mazama gouazoubira* (Fischer, 1814)	Gray Brocket	Fr/He
Bos taurus Linnaeus, 1758	Cow	He
Carnivora		
Cerdocyon thous (Linnaeus, 1766)	Crab-eating Fox	Om
Nasua nasua (Linnaeus, 1766)	South American Coati	Fr/Om
Birds		
Gruiformes		
Aramides saracura (Spix, 1825)	Slaty-breasted Wood-rail	Om
Galliformes		
Penelope obscura (Temminck, 1815)	Dusky-legged Guan	Fr/Om

Columbiformes		
Columbina talpacoti (Temminck, 1811)	Ruddy Ground-dove	Gr
Passeriformes		
Cyanocorax chrysops (Vieillot,1818)	Plush-crested Jay	Om
Turdus rufiventris (Vieillot, 1818)	Rufous-bellied Thrush	In/Om
Turdus leucomelas (Vieillot, 1818)	Pale-breasted Thrush	In/Om
Saltator similis (D'Orbigny & Lafresnaye, 1837)	Green-winged Saltator	Om
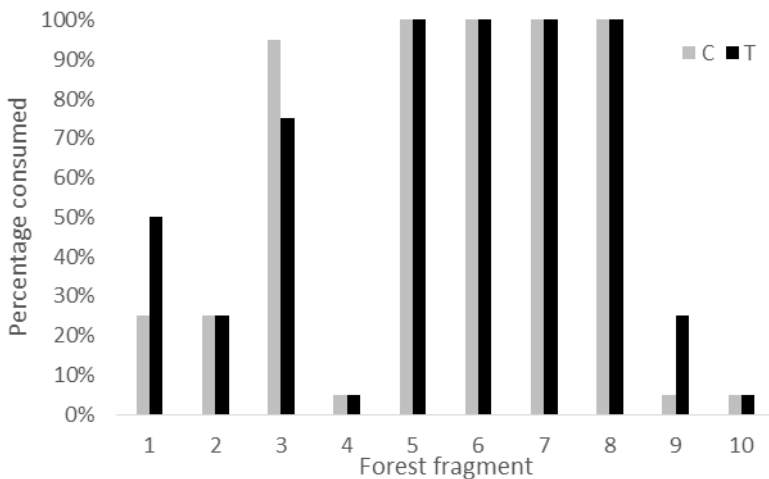
Figure 1: Consumption of 40 maize cobs (20 conventional and 20 transgenic) in ten Atlantic Forest fragments surrounded by transgenic maize crops in Campos Novos, Southern Brazil. C: Conventional maize, T: Transgenic maize.

Table 2: Average consumption of two maize cobs (conventional and transgenic) by native animals in ten Atlantic Forest fragments surrounded by transgenic maize crops in Campos Novos, Southern Brazil.

Fragment	Conventional maize consumed (%)	Transgenic maize consumed (%)	Differences (d)
1	8.75	15.00	6.25
2	2.50	2.50	0
3	97.50	97.50	0
4	97.50	97.50	0
5	97.50	97.50	0
6	91.25	73.75	-17.50
7	97.50	97.50	0
8	15.00	50.00	35.00
9	2.50	8.75	6.25
10	2.50	2.50	0
$\bar{x}$	51.25	54.25	3

Discussion

There were no differences in consumption by animals when conventional or transgenic maize was offered. It shows that there are no food preferences or some kind of unpalatability for animals. The difference in maize consumption is possibly due to the availability of resources present in each forest fragment. The higher consumptions possibly indicate few available resources in the fragments. Maize is an abundant food resource during some periods, and for consumers with broad diets - omnivorous - a trade-off between food availability and foraging efficiency is required (Futuyma and Moreno 1988). In environments with high levels of environmental degradation there is a growing number of omnivorous birds and possibly of insectivorous less specialized birds, (Motta-Júnior, 1990), as observed in this study.

It is already known that small granivorous mammals increase in abundance near maize plantations and cornfields, which modify the structure of small tropical mammal communities by providing a complementary resource (i.e. maize) (Braga et al., 2015). These possibly change the entire food chain within these landscapes, and the opportunistic medium sized mammals may have benefited from these areas with the available complementary resource (small mammals and maize). Opportunistic mammals (e.g. *D. novemcinctus*, *C. thous*, *D. albiventris* and *N. nasua*) were recorded in the majority of fragments surrounded by plantations, and generalist mammals were the most

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photographed species (e.g. *M. gouazoubira, D. novemcinctus* and *N. nasua*). These species are capable of using transformed habitats and utilize cultivated lands near forest areas (Pinder and Leeuwenberg, 1997; Andrade-Núñez and Aide, 2010).

Cornfields provide a complementary resource (Ries and Sisk, 2004) to small rodents in fragmented tropical forests (Braga et al., 2015), and also provide a complementary resource for medium and large opportunistic mammals in Atlantic Forest fragments. The generalists, for example, tend to sample new food sources in order to survive (Krebs and Davies, 1993). Within the study region, at least 21 native mammals use forest patches surrounded by maize crops (Campos and Hernández, 2015b). The effects along the food chain could be harmful to these native mammals, which may suffer from the latent effects of transgenic crops, such as those observed with laboratory rats (Séralini et al., 2014).

The period before the maize harvest coincides with the reproduction of mammals. Lactation is generally considered the most expensive aspect of reproduction for a female mammal (Millar, 1977; Gittleman and Thompson, 1988). Thus, the reproductive output is limited by energy resource, and the maize crops increase the available food resource in these areas for the females, as well as providing resources for their young.

Nowadays, the mode of action of GM is most discussed than in the early 1990s, when the Bt plants were first developed and promoted (Vachon et al., 2012). The negative effects of genetically modified plants on non-target invertebrates were found in certain cases (Obrist et al., 2006; Rosi-Marshall et al., 2007; Hilbeck et al., 2008; Wolfsbarger et al., 2008; Chambers et al., 2010; Duan et al., 2010; Then, 2010; Holderbaum et al., 2015). These effects on wildlife along the food chain may also be occurring, such as changes in the abundance of dung beetle communities in Atlantic forest fragments surrounded by transgenic maize (Campos and Hernández, 2015a, 2015b). Dung beetles use feces of mammals as a food resource, and could be exposed to transgenic material through mammals feces (Lutz et al., 2005; Paul et al., 2010; Guertler et al., 2010).

In conclusion, consumption and food preferences by native animals are not related to the type of maize in Atlantic Forest fragments surrounded by maize, and the search and exploitation were similar between transgenic and conventional maize. If transgenic crops have some unknown latent effect in animals that feed on this type of food,

wildlife will also suffer such effects, since they do not differentiate between them.

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Apêndice Capítulo 2: Fotografias da realização do experimento em fragmentos de Mata Atlântica na região de Campos Novos, Santa Catarina, durante o ano 2014

Quatro espigas de milho (2 convencionais e 2 transgênicas) dispostas em frente a armadilha fotográfica.

Marcação de espigas de milho (2 convencionais com uma marca vermelha e 2 transgênicas, com duas marcas) pouco consumidas após 20 dias de exposição.

Pesquisadora checando a armadilha fotográfica.

Indirect exposure to Bt maize through pig feces causes behavioral changes in dung beetles

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Abstract

Genetically modified (GM) Bt plants currently represent a highly adopted alternative for pest control in agricultural crops. However, their safety to non-target organisms has been an unsolved issue. Non-target organisms associated to nutrient cycling in natural and agricultural systems, such as dung beetles, use feces of mammals as a food resource, and could be exposed to Bt-plant material through feces of live-stock fed with Bt-crops. Thus, the aim of this study was to assess whether indirect exposure to transgenic Bt-maize MON810 can reduce fitness in dung beetles. Two dung beetles species, *Canthon rutilans cyanescens* and *Coprophanaeus saphirinus*, were reared under laboratory conditions and fed with pig feces during three months, using two treatments/diets: feces of pigs fed transgenic maize and of pigs fed conventional maize. Organic matter incorporation in the soil per male-female pairs of *C. rutilans* was similar between GM and conventional treatments, as was their reproductive success, but beetles fed with feces from transgenic maize produced more brood balls. In another trial regarding the incorporation of organic matter in the soil, *C. saphirinus* fed with feces derived from conventional maize showed greater ability to bury food resource in comparison with GM fed ones. In an olfactometer test, the time to reach the food source was longer for individuals of *C. rutilans*, previously fed with transgenic feces during one month, than individuals fed with conventional feces. We hypothesize that differences found in dung beetles' ability represent potential indirect effects of transgenic maize through the food chain, and may also affect ecological functions of these organisms in natural habitats, by means of reduced beetle efficiency in removal and burial of fecal masses.

Keywords: non-target, GMO, fitness, Scarabaeinae, nutrient cycling

Introduction

The agricultural use of transgenic or genetically modified (GM) plants currently may represent an alternative for insect pest control. Crops such as soybeans, corn, cotton, potato and tobacco, among others, have been genetically modified to express genes derived from *Bacillus thuringiensis* Berliner (Bt) (James, 2003). Bt genes (*cry*) code for insecticidal proteins/toxins, also called δ-endotoxins (Bravo et al., 2007). Such GM plants are used on a commercial scale in various countries, including Brazil. Native Bt toxins are known to be highly specific to target organisms and are generally stated that not to affect non-target organisms (Schuler et al., 1998, Betz et al., 2000). However, this notion has been recently disputed (Ramirez-Romero et al., 2008, Bøhn et al., 2008, Vachon et al., 2012, Van Frankenhuyzen, 2013, Bøhn et al., 2016). The specificity and mode of action of Bt toxins as pest controllers in Bt plants depends on the specific toxin derived from *B. thuringiensis*, which produces several different toxins that present activity on the digestive system of some insect families. Specifically, one such toxin, the Cry1Ab protein, is regarded as specifically toxic to certain lepidopteron species, and is expressed by *B. thurigiensis* only during sporulation, in crystalline inclusions of an inactive pro-toxin, which needs to be cleaved under specific conditions, such as high pH and presence of certain proteases, and to find specific receptors in the gut of target organisms to become active and toxic (Bravo et al., 2007). In Bt plants such as MON810 maize, however, the recombinant Cry1Ab (rCry1Ab) toxin is expressed continuously and in different quantities in the various tissues of the plant throughout the life cycle (Székács et al., 2010a), creating a different scenario for non-target organisms than that occurs when the bacteria is used in pesticide sprays. In addition, MON810 maize carries a recombinant cry1Ab gene that codes for a pre-activated 91-kD, instead 130-kD by the native cry1Ab gene (CERA, 2015). Moreover, since the inserted transgene in MON810 event is truncated, a truncated rCry1Ab toxin is expressed (Hernández et al., 2003).

Several studies reported no adverse effects of Bt maize on non-target organisms (e.g.: Marvier et al., 2007, Wolfsbarger et al., 2008, Naranjo, 2009), so Bt–plants seem to be less harmful than chemical insecticides (Marvier et al., 2007, Naranjo, 2009). However, negative effects in non-target invertebrates have also been found in several studies (Obrycki et al., 2001, Harwood et al., 2005, Zwahlen and Andow, 2005, Hilbeck and

Schmidt, 2006, Obrist et al., 2006, Rosi-Marshall et al., 2007, Hilbeck et al., 2008, Bøhn et al., 2008, Wolfsbarger et al., 2008, Chambers et al., 2010, Duan et al., 2010, Then, 2010, Holderbaum et al., 2015).

Negatives effects also been found in dung beetles (Campos and Hernández, 2015a, 2015b*),*which are detritivorous organisms that use mainly feces of mammals as food resource and are strongy associated to food chain (Halffter and Matthews, 1966, Estrada et al., 1999, Andresen and Laurance, 2007). The effects observed in dung beetles may be related to the presence of transgenic DNA or proteins in mammals' feces used as resource, because transgenic Bt DNA and proteins can pass intact or as biologically significant fragments through the gastrointestinal tracts of mammals or birds (Lutz et al., 2005, Paul et al., 2010, Guertler et al., 2010).

Dung beetles promote removal and incorporation of organic matter in the soil, the construction of tunnels by some of these beetles allows greater aeration and moisture in the soil as well as the incorporation of nutrients present in feces, promoting nutrient cycling and regulation of physiochemical properties of the soil (Halffter and Matthews, 1966, Halffter and Edmonds 1982, Hanski and Cambefort 1991, Nichols et al., 2008). The nesting behavior of dung beetles is closely related to the use of food resources, and according to how the resource is used dung beetles are divided into three functional groups: rollers, tunnelers or dwellers (Halffter and Edmonds, 1982).

The hypothesis of this study is that dung beetles fed pig feces, which previously fed transgenic maize, reduce their fitness and alter some behavioral characteristics, due to a non-lethal toxic effect of the rCry1Ab protein produced by MON810 *Bt* maize. The aim of the study was to test whether indirect feeding of transgenic maize through the pig feces can cause loss or decrease of fitness in dung beetles that fed these feces.

Materials and Methods

Experiments with dung beetles, were designed to evaluate beetle's ability to bury organic matter and detect resources. The following steps were carried out in order to develop these experiments:

1) To obtain maize grains without pesticides, transgenic maize (GM event MON810, AG 5011 YG hybrid, expressing the rCry1Ab protein) and conventional maize seeds (AG 5011 hybrid, a non GM counterpart) were planted in the Ressacada/UFSC experimental station in

Florianópolis, south Brazil, during December 2012 and January 2013. The only management practice conducted was the addition of urea fertilizer after sowing the seeds. To avoid crossings between GM and non-GM maize, a spacing of 500 m between the two types of maize and a four-week interval between plantations was used. Harvest was done manually, the cobs were dried at 40 ºC for 72 h, grains were threshed, ground and passed through a 2 mm sieve. The resulting maize meal was used as the basis of two types of pig feed - GM and conventional - hereafter denominated as GM and non-GM treatments. Pig feed was prepared in a horizontal mixer and consisted of 60% maize, 30% organic soybean and 10% suplement (Supermix L-15 Vitamix). Pig feed was made either with conventional maize (non-GM) or transgenic maize (GM). Previously, the detection of transgenic DNA in both maize type was done by means of PCR (polymerase chain reaction), using the 35S promoter as marker sequence and the zein gene as endogenous reference sequence (data not shown).

2) To obtain feces to feed dung beetles, ten recently weaned piglets were raised in the Ressacada/UFSC experimental farm. All piglets were born from different parents and were housed individually. Five piglets were fed with non-GM feed and five piglets were fed with GM feed, during three weeks in February 2014. In the first week, iron oxide was added to the feed, to check digestibility before beginning the experiment. After this adaptation period, feces were collected twice a day during two weeks, stored individually and frozen for later use. Detection and quantification of rCry1Ab protein in pig feces was carried out in the Proteomics Laboratory - CCA/ UFSC, using a Cry1Ab enzyme linked immunossorbent assay (ELISA) kit (QualiPlate Kit for Cry1Ab/ Cry1Ac- ENVIROLOGIX), following the manufacturer's instructions, with adaptations to allow for analysis of pig feces and quantitative results: 50 mg of pig feces were used for all feces samples, and a serial dilution of trypsinated Cry1Ab core toxin from *Bacillus thurigiensis* (0, 10, 20, 40 and 80 ng mL^{-1}) was used to construct a standard curve. Total protein was extracted from 50 mg feces with 250 µL extraction buffer (PBS + Tween 20 (0.5%) and used to quantify Cry1Ab protein. Results were presented as ng of rCry1Ab protein g^{-1} of feces fresh weight.

3) Collection of beetles **-** Living dung beetles of four abundant species from Scarabaeinae subfamily were sampled inside native forest fragments in Santa Catarina, using pitfall traps during the summer of

2015 and 2016, in approximately 30 days of sampling, with the traps being exposed 24 or 48 h. The traps contained soil and dog feces baits, in order to attract dung beetles. Live insects caught in traps were taken and reared in the Laboratory of Terrestrial Animal Ecology (LECOTA/UFSC).

Dung beetles rearing were carried out during the summer of 2015 and summer of 2016 in standard laboratory conditions: 27±1°C, 60±10% relative humidity and photoperiod of 12 h. Four species of Scarabaeinae were utilized in the experiments: two rollers - *Canthon rutilans cyanescens* Harold, 1868 and *Canthon angularis* Harold, 1868 - and two tunnelers - *Coprophanaeus saphirinus,* Sturm, 1826 and *Phanaeus splendidulus* (Fabricius, 1781). Beetles were fed during three months with pig feces and maintained in terrariums for breeding. The terrariums were 30 cm high and 20 cm in diameter, containing soil and food according to the treatment - GM or non-GM. The experiments were divided into two types: experiments of foraging behavior with olfactometry measures and experiments on the incorporation of organic matter in the soil and nesting.

In 2015, for experiments of removal and burial of organic matter and nesting, adults of two species, one roller - *C. rutilans* - and one tunneler - *C. saphirinus* - were used. Five grams of resource (feces) were offered twice a week for beetles of both species during three months. For *C. saphirinus*, the amount of feces buried was evaluated using two individuals per terrarium. A total of 11 terrariums were used in the GM treatment and ten terrariums in the non-GM treatment. For *C. rutilans*, the ability of a couple of beetles to food balls in the soil was quantified. Experiments were conducted in terrariums (30 cm high and 20 cm in diameter). Five terrariums were used per treatment (GM and non-GM). Reproductive success (F1) was evaluated and brood balls were counted.

Behavioural experiments were carried out in March 2016, using an olfactometer. A four-arm olfactometer was designed to test the possible effects of transgenic maize on the olfatory detection of dung and in the locomotion capacity of dung beetles. The olfactometers consisted of a central arena with four exits (described in detail in Verdú et al. 2007). The central arena consisted of a plastic truncated cone (60 cm superior radius and 40 cm inferior radius) with sterile dry vermiculite as substrate and four 5-cm-diameter holes to attach the tubes (arms) containing the plastic containers with test samples at the ends. The plastic containers were made to capture the beetles that responded positively to the tested

resources. Air, which had been passed through an activated charcoal filter, was drawn into the plastic containers of the olfactometer. In the centre of the arena, there was a 12-cm hole to attach a tube with an air-out ventilator. Complete sealing of the system was ensured with adhesive tape used to join all connections. The temperature in the experiment room was maintained at 26º-27ºC. The tubes were wrapped in aluminum foil to prevent light from entering. Odour sources were randomly placed in the olfactometer in each trial.

Three species were used in the experiment: *C. rutilans* (50 individuals per treatment), *P. splendidulus* (10 individuals in GM treatment and 11 in non-GM treatment) and *C. angularis* (13 individuals in GM and 18 in non-GM treatment). Before begining the experiment, beetles from all species were kept in terrariums and fed with pig feces (non-GM or GM) during one month. Forty eight hours before the olfactometry experiment, food was removed in order to increase beetle attraction to resources. For this test, two containers with odour sources (pig feces) as well as two empty containers were used. After placing the beetles in the arena, a 10 min window was set before starting the experiment in order to allow the beetles to adapt to the new conditions. After that, all beetles were given 24 h to select a container. Results were recorded after six, 12 and 24 h. After 24 h all the dung beetles were removed. Beetles from each of the six species-treatment combinations (*C. rutilans*, *P. splendidulus*, *C. angularis* combined with GM or non-GM treatments) were placed in the arena at independent times.

Data from laboratory experiments conducted for each species fed GM or non-GM derived feces were analyzed with ANOVA (Analysis of Variance) or GLMs (Generalized Linear Models). A GLM with binomial distribution, adequate for dichotomic responses (e.g. beetle found or did not find the resource) was used for data from the olfactometer experiment, ANOVA and GLM with Poisson distribution (count data) were used for data from removal and burial of organic matter and nesting, respectively.

Results
Concentration of rCry1Ab in the feed diet
Transgenic DNA was detected by PCR only in the transgenic maize. Likewise, the rCry1Ab protein was only detected by means of ELISA in the feces of pigs fed transgenic maize, and no traces were detected in feces of pigs fed conventional maize. The average concentration of

rCry1Ab protein in the feces of pigs fed transgenic maize was 304± 45, 96 ng/g (Table 1).

Table 1: Concentration of Cry1Ab protein in the feces of pigs fed transgenic maize, as estimated by ELISA.

Pig feces samples	Cry1Ab ng/g
Trans1	363,33
Trans2	322,50
Trans3	261,67
Trans4	240,00
Trans5	332,50

Experiments on organic matter incorporation in the soil and nesting by dung beetles

Pairs of *C. saphirinus* buried on average 23.01 ±0.30 (mean ± SD) grams of GM resource and 28.40 ± 5.95 (mean ± SD) grams of non-GM per month, evidencing that beetles buried significantly less GM resource (z value= 3.31, p=0.0009). Similar pattern was maintained in the second (z value = 2.56, p= 0.010) and third month (z value = 4.05, p= 0.0005) (Fig 1). However, the incorporation of organic matter by a pair of *C. rutilans* through food balls was similar in GM and non-GM treatments (F=0.231, p=0.631), with 2.78 ± 0.36 food balls per pair in non-GM treatment and 2.68 ± 0.16 food balls in GM treatment per month.
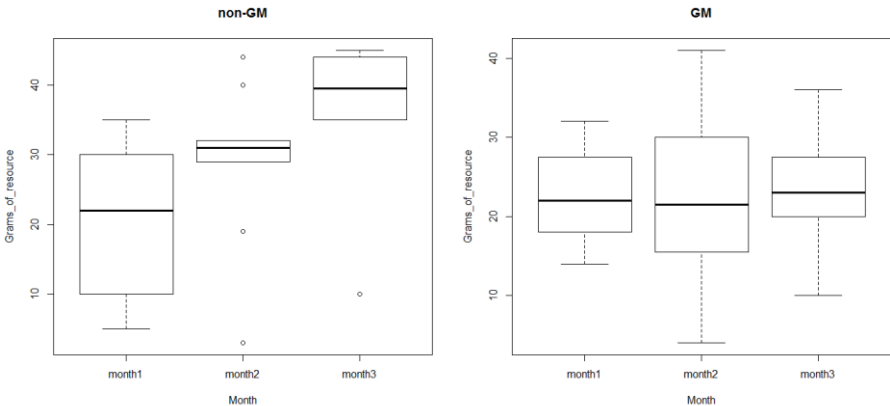
Figure 1: Amount of feces buried by *C. saphirinus* (in grams) in three months of experiment under diets of GM and non-GM derived pig feces.

The transgenic treatment resulted in more brood balls (z value= 2,442, p=0.014) (Fig 2). On average, couples under the GM treatment produced 2.10 brood balls, while couples under non-GM treatment produced 1.66 brood balls. The fertility of *C. rutilans*, the relation between the emerged beetles/brood balls was similar (z value= 0,199, p=0.84) between GM and non-GM treatments). The reproductive success (F1) was also similar: five individuals emerged in non-GM treatment and six individuals emerged in GM treatment along three months. The average time to emergence of *C. rutilans* was 44.5± 3.5 days and 45.5 ± 1.5 days for GM and non-GM, respectivelly.
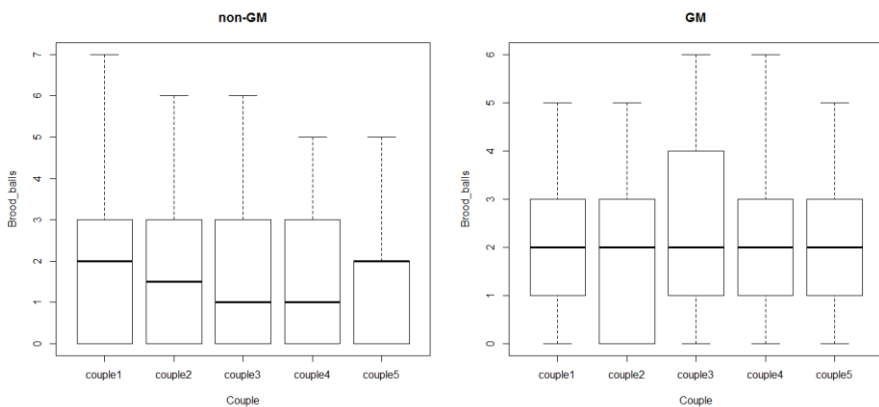
Figure 2: Number of brood balls of *C. rutilans cyanescens* in three months of the experiment under GM and non-GM treatments.

Effect of transgenic maize-derived feces on foraging behaviour

Olfactometer tests showed a significant negative effect of the GM treatment (z value= 2.42, p=0.0153*)* on foraging sucess of *C. rutilans*. The time required for detection and arrival at the food resource was increased in *C. rutilans* fed GM maize-derived feces (z value= 3.07, p=0.00214*)*, with significantly more beetles under the non-GM treatment reaching the resource in 6 hours. In 24 h beetles under both treatments arrived in the resource equally (Fig 3). The other two species did not show differences between GM and non-GM treatments (*P. splendidulus (*z value= 1,25, p= 0.210) and *C. angularis* (z value= 1.36 , p= 0.173)). It was also assessed whether the type of feed changed the locomotion capability of dung beetles - i.e. whether dung beetles remained in the arena or if they went in the containers - but the

differences were not significant for any of the three tested species: *C. rutilans* (z value= 1.56, p= 0.11) *P. splendidulus (*z value= 1.51, p= 0.13) and *C. angularis* (z value= 0.93 , p= 0.34).
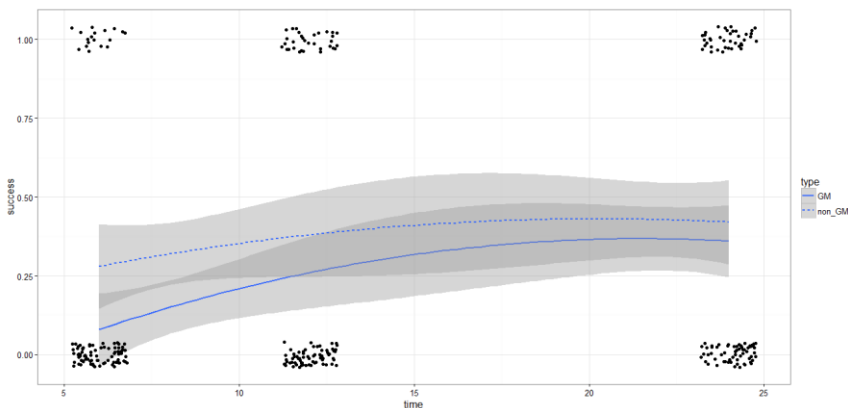
Figure 3: Binomial regression for time required to detection and arrival at food resource of *C. rutilans*, during 24 hours observation at the olfactometer test under GM and non-GM treatments.

Discussion

    Previous studies have already reported the presence of transgenic DNA fragments in the intestinal tract of pigs (Klotz et al. 2002, Chowdhury et al. 2003, Reuter and Aulrich, 2003). The detection of rCry1Ab protein in mammal's feces was also observed in other studies (Chowdhury et al. 2003, Einspanier et al. 2004, Lutz et al. 2005, Guertler et al., 2008, Paul et al., 2010, Zdziarski et al. 2014). The levels of rCry1Ab protein detected in pig feces in the present study (304 ± 45.96 ng/g) were similar to levels reported in previous research with pigs (Chowdhury et al. 2003). In pigs, Cry protein fragments are detectable but are progressively reduced in size as they travel in the gastrointestinal tract (Chowdhury et al., 2003). It is notable that many farm animals generally have a high proportion of transgenic maize in their diets, and if Cry proteins from Bt crops are present in livestock feces it can reach organic matter decomposers such as dung beetles. Thus, the fecal excretion of rCry1Ab protein into the soil may be an additional risk concern (Chowdhury et al., 2003a).

    Subtle, non-lethal effects were detected in the experiments herein described, but overall, not all tested species were equally affected. The

time required for *C. rutilans* beetles to reach the resource was higher in the GM treatment and the quantity of buried resource was higher in the non-GM treatment. In addition, dung beetles fed feces derived from GM maize produced more brood balls, what could indicate a strategy of energy allocation for reproduction under higher stress, a response that was previously observed in other non-target arthropods exposed to Bt-maize material (Bohn et al., 2008, Holderbaum et al., 2015). In bioassays with *Daphnia magna* fed GM maize was detected a resource allocation to production of resting eggs and early fecundity (Holderbaum et al., 2015).

Among non-target organisms of recombinant Bt proteins, dung beetles are an important group in terms of diversity, abundance, biomass and functional relevance within the dung pat communities (Nichols et al., 2008). It is well know that the structure of dung beetle communities is influenced by high competition for scarce and ephemeral food resources (Hanski and Cambefort, 1991, Simmons and Ridsdill-Smith, 2011). If the time required to reach resources is greater for beetles exposed to pig feces derived from GM corn, sensitive species, such as *C. rutilans*, may be more easily outcompeted by unaffected species, with fewer individuals reaching the resource. Despite the tunnelers are the most efficient in the removal and burial of resources, the rollers are very abundant in southern Brazil (Campos and Hernández, 2015a, Da Silva and Hernández, 2014). Consequently, such effect could potentially impact the ecological functions provided by dung beetles, such as removal and burial of organic material (Braga et al., 2013).

Many experimental ecotoxicology studies have focused on breeding behavior and survival rates, but to the best of our knowledge, no data are available on the indirect effects of transgenic Bt-maize in dung beetles. Importantly, differences in dung beetles communities were detected in forest fragments surrounded by transgenic maize (Campos and Hernández, 2015a, 2015b). In a field study (Campos and Hernández, 2015a) detected a decrease in tunneler beetles, and a delay in the time to reach the resource, as was observed in this study under laboratory conditions, could explain the difference in fitness of the affected species. In addition, after two years in the same study region (Campos and Hernández, 2015b), a decrease was observed in the whole of dung beetle community in forest fragments surrounded by GM maize. The results of the experiment herein reported supports that GM maize was a causal factor of that previous study. Moreover, subtle effects, such as time to

reach the resource, can generate cascade effects and the whole community can be affected. Thus, changes in soil species dynamics can not be excluded as an biohazard of GM Bt varieties.

A recent study shows that ivermectin decreases the sensorial and locomotor capacity of dung beetles (Verdú et al., 2015), an example of dung contamination and cascade effects in dung beetles. Deficiencies in competitive capacity of dung beetles can affect their functions in natural environments, making them less efficient in the removal and burial of fecal masses. The amount of buried resource was higher for tunneler dung beetles in the non-GM treatment. Dung beetles reduce and incorporate fecal masses in the soil, playing an important ecological role in nutrient cycling, organic matter decomposition and assistance in soil aeration via tunnel building (Nichols et al., 2008). The efficiency of functions performed by dung beetles (i.e removal and burial of fecal masses) is more effective among tunnelers (Halffter and Edmonds 1982, Anduaga and Huerta 2007). In general, the tunnels of pararacoprids, such as *C. saphirinus*, are larger, deeper and cause greater soil movement (Halffter and Edmonds 1982). Particularly, *C. saphirinus* is a large tunneler, very frequent in Atlantic forest of south and southeast of Brazil.

Females of *C. rutilans* fed with transgenic corn-derived feces produced more brood balls, despite the reproductive success being the same in the GM and non-GM treatments. Female dung beetles investing a large amount of energy to build brood balls containing a single egg, and environmental and biological variation can result in the optimal reproductive strategy (Reaney and Knell, 2010). In addition, female's reproductive investment has large effects on offspring quality. Moreover, an increased investment in reproduction may occur in order to maximize reproductive success: this is called "terminal investment" (Clutton-Brock 1984).

Non-lethal effects demonstrated by the use of GM corn in this research can render dung beetles less competitive to reach ephemeral resources and increase predation, resulting in decreased populations. Thus, the results explains the loss of diversity previously observed in dung beetle communities within fragments of native forest in south Brazil (Campos and Hernández, 2015b), which can result in decreased ecosystem services provided by these beneficial insects.

Fitness is a crucial population feature modulates by natural selection, which usually increase the adaptive values. The cropping domesticated

GM varieties carrying recombinant Cry toxins nearby forest fragment provoke an environmental perturbation that causes the decrease of fitness of population of non-target species. Thus, previously of release of GM varieties, regulatory agencies should require also this type of studies.

Acknowledgments

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Apêndice Capítulo 3: Fotografias das etapas cumpridas para a realização dos experimentos
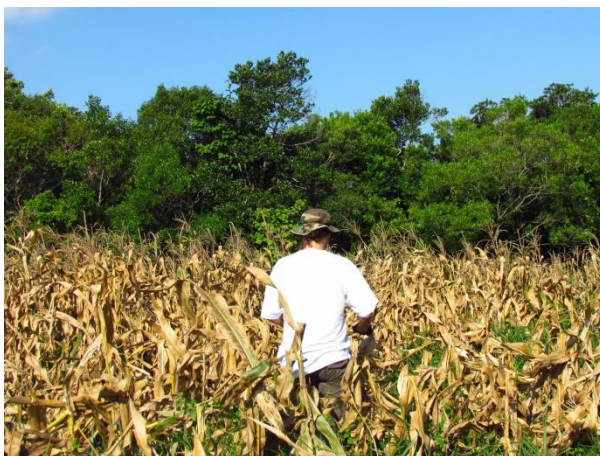
Colheita manual do milho transgênico.

Pesquisadora debulhando o milho para ser utilizado na ração.
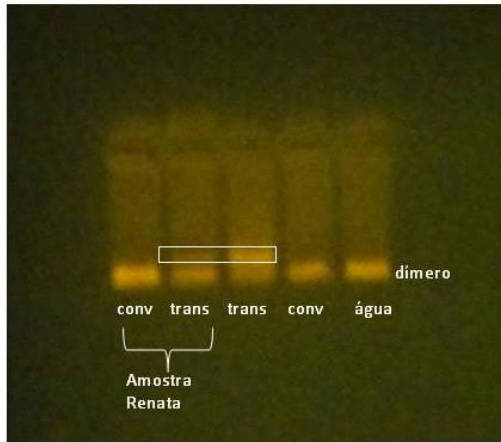
PCR do milho transgênico.

Visão geral dos recintos dos porcos e pesquisadora fazendo a manutenção diária.

Recintos individualizados para cada porco.

Dez porcos utilizados para obtenção das fezes nos dois tratamentos.
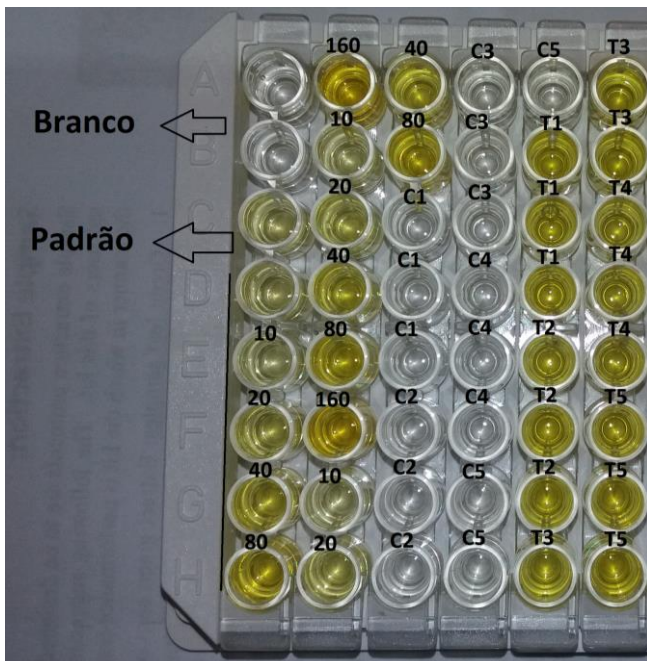
Microplaca do teste ELISA: Os dois primeiros poços contêm o branco, seguido de dois poços com a proteína padrão do teste, seguidos pela diluição da proteína purificada Cry1Ab (10, 20, 40, 80 e 160 ng mL^{-1}), as cinco amostras de fezes tratamento não transgênico (C1, C2, C3, C4, C5) e as cinco amostras de fezes no tratamento transgênico (T1, T2, T3, T4, T5).
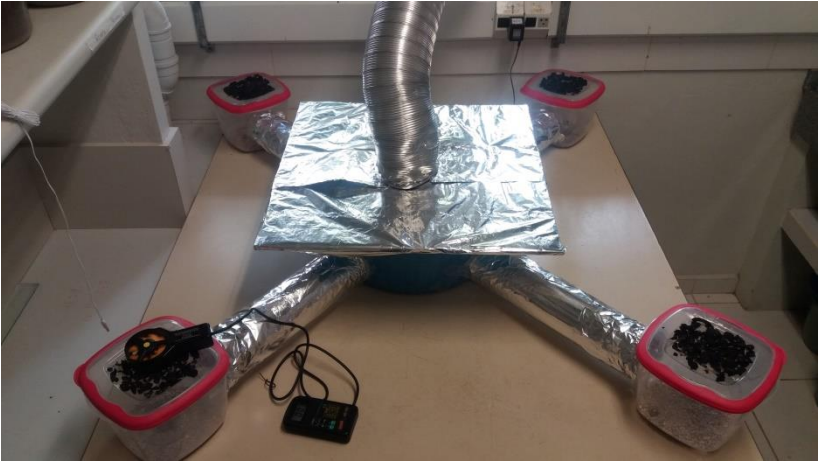
Olfatômetro utilizado no experimento de atração dos besouros.

Effects of indirect ingestion of Bt maize in the ecophysiology of *Canthon rutilans cyanescens* (Coleoptera: Scarabaeidae)

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Abstract
Dung beetles act in nutrient cycling, an ecosystem service of their feeding and reproductive behavior. Feces of large mammals are the main food source of dung beetles. The hypothesis that GM maize (Bt), used against agricultural pests, can affect dung beetles via consumers (mammals) along the food chain led us to evaluate if there are ecophysiological changes in dung beetles that fed feces of pigs fed with transgenic maize. Therefore, three ecophysiological experiments to evaluate the detection of changes in the perception of chemical stimuli in the environment were developed. In electroantennography experiment, no differences were detected between the two treatments: beetles was fed with feces of pigs who had consumed transgenic maize and another group with feces of pigs who had consumed conventional maize . In the experiments of respirometry and phenoloxidase activity an increased evidence of the activity of the immune system was detected: increase in the emission of CO_2 during respiration and increased phenoloxidase activity in beetles that consumed a diet with transgenic. This can be understood as a way to maintain the balance of the immune system in a harmful environment. We propose that GM maize is acting as an environmental stressor for non-target organisms, such as dung beetles.

Keywords: behavior, dung beetles, GM, physiology, immune system

Introduction
The use of transgenic or genetically modified (GM) plants that express genes with insecticidal activity, represent an alternative to control insect pests. However, there are uncertainties about the

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possibility of transgenic plants affect non-target organisms, which have attracted the interest of researchers and regulatory agencies. Most transgenic plants resistant to insects express genes derived from the bacterium *Bacillus thuringiensis* (Bt). The entomopathogenic activity of this microorganism is due to the presence of an insecticide core of this protein in crystal form. After ingestion by susceptible insects, the crystals are dissolved in slightly acidic pH (e.g. Coleoptera) or alkaline (e.g. Lepidoptera and Diptera) of the middle intestine of the insect and the protoxin are released. Protoxins are carried by intestinal proteases into smaller peptides, which bind to specific receptors in the epithelia, promoting the osmotic disruption of epithelial cells lining the gut, causing the death of the insects. Death may also occur due to a second cause associated with the first, bacterial multiplication in the hemolymph, determining a septicemic process (Gill et al., 1992; Monnerat & Bravo, 2000). The precise details of how endotoxins bind to gut cells to kill insects are poorly understood (Ferre & Van Rie, 2002).

Negative effects of GM crops on the associated fauna along the food chain are poorly known (Obrycki et al., 2001; Lovei et al., 2009). When the transgenic plants were developed scientists stated that the toxins were highly specific and did not affect non-target organisms (Schuler et al., 1998; Betz et al., 2000). However, this is not true, because the detection of adverse effects in many species (Van Frankenhuyzen, 2013) and currently the mode of Bt toxins action is subject to more uncertainties than in the early 1990s, when the Bt plants were first developed and promoted (Vachon et al., 2012).

The dung beetles (Coleoptera: Scarabaeinae) participate actively in the nutrient cycling using decaying organic matter in feed and reproduction, and are extremely important in the functioning of tropical ecosystems. Most species feed on feces or carcasses (scavengers), especially of mammals, thus being intrinsically associated with animals that produce their food resource (Halffter & Matthews, 1966; Halffter & Edmonds, 1982). Dung beetles are grouped into three functional groups depending on resource allocation behavior: rollers or telecoprids, tunnelers or paracoprids, and dwellers or endocoprids. *Canthon rutilans cyanescens* is a ball-roller species that inhabits forests, and is a non-target organism to GM maize (Bt).

Olfactory response in dung beetles

In dung beetles, the attraction for food is mediated by compounds released by excrements, decomposed bodies of vertebrates or decaying fruit (Favila et al., 2012). To find the resources, the olfactory system plays an important role in detect various chemical stimuli in the environment. Insects have specialized sensory organs that are located in the antennae and the chemical messages are captured by them (Hansson & Anton, 2000). The detection of chemical signals (chemoreception) of the environment can be through the olfactory or gustatory system, and the signs are mainly detected by the antenna, maxillary palps and labial palps. Odors are detected by olfactory receptor neuron (ORN) that are situated within the olfactory hairs (olfactory cells), which are located in the antennae and palps. The ORN's detect specific odors with varying levels of specificity and sensitivity (Rojas & Malo, 2012).

The dung beetles developed chemical communication for the detection of food, sexual attraction and recognition, and developed chemical mechanisms of defense against competitors and predators (Favila, 2001). Changes in the antennal responses of beetles could prevent detection of various chemical stimuli in the environment. A recent study shows, for example, that ivermectin decreases the olfactory ability of dung beetles (Verdú et al., 2015).

Respirometry thermolimit

In dung beetles, studies show that the maximum and minimum limits of thermal tolerance range between 16ºC and 45ºC, respectively. The maximum threshold temperature is close to the thermal shock, which raises the body temperature maintenance behavior (Verdú et al., 2006).

Increases in environmental temperatures induce exponential increases in oxygen demand at the organism, resulting in reduced physiological performance and eventually death (Brown et al., 2004). In Deltochilini (e.g. *Canthon*), species fly with its elytra closed, regulating heat excess according to the degree of separation between these and the abdomen and this individuals can fly in or out of forests with great skill (Verdú & Lobo, 2008).

In determinations of insect respiratory critical thermal limits, the combined use of respiratory measurements and thermography generate more conclusive interpretation of respiratory traces.

Immune system

In insects, innate immunity is well developed and allows a quick response to infectious agents. The main body of intermediary metabolism in insects is the fat body, responsible for synthesis and for providing several hemolymph components. There is evidence that fat body has an important role in immune response, the excessive intake of food ingested by the insects during the larval stage is stored in this tissue, and some peptides, such as defensin, are produced and released into the hemolymph (Zanetti et al., 1997; Hoffmann et al., 1999). In this immune system, phenoloxidase (PO) is the most important component and its activity is often used as a measure of the ability of the immune defense of individuals (González-Santoyo & Córdoba-Aguilar, 2012; Verdú et al., 2013). Furthermore, Bt resistance has been associated with higher phenoloxidase activity in Lepidoptera (Rahman et al., 2004; Ma et al., 2005).

This study is based on the assumption that dung beetles that fed mammalian feces, which fed on GM maize, were exposed to plant material and toxins derived from GM maize, as the transgenic DNA and proteins can pass gastrointestinal tracts of mammals or birds, intact or in biologically significant fragments (Lutz et al., 2005; Guertler et al., 2010; Paul et al., 2010), and this may cause ecophysiological changes in dung beetles, disabling the detection of various chemical stimuli in the environment.

The purpose of the study was to examine possible impacts of transgenic plants (Bt) on non-target organisms associated with mammals through the food chain. The objective was to determine if there are differences in dung beetle antennal responses, respiration rates, and the phenoloxidase (PO) activity in individuals subjected to two treatments: beetles fed with pork feces with conventional maize, and fed with pork feces with transgenic Bt-maize.

Materials and Methods
Experimental insects

Individuals of the dung-roller beetle, *Canthon rutilans cyanescens* Harold, 1868, were captured in a tropical forest fragment in Florianópolis, Brazil, in May 2015. Specimens were reared in an insectarium (at 27ºC±1ºC, 70±10% RH, with a photoperiod of 12 hours). They were fed with swine feces during one month, some with feces of pigs that were fed with Bt maize (Cry1ab) and others with feces of pigs that were fed with conventional maize. Then they were brought alive to

Alicante University (CIBIO), Spain, and after a period of adaption (five days) the tests were carried out.

Electroantennogram recordings

Electroantennogram signals were recorded with an EAG system (Syntech, Kirchzarten, ®Germany) consisting of a universal single ended probe (Type PRG-2), a stimulus controller (CS-55), a data acquisition interface board (Type IDAC-02), and a stimulus air controller (CS-55 stimulus controller). The antennae of *C. rutilans cyanescens* were excised, inserted into small droplets of electrode gel (Spectra 360, Parker Laboratories, Fairfield, NJ, USA) and mounted individually between the electrodes in an antenna holder under a purified air flow (500 ml/min) (Supplementary Figure S1). A Syntech PC-based signal processing system was used to amplify and process the EAG signals. The signals were further analysed using the EAG 2000 software (Syntech, Kirchzarten, Germany).

Stimulation tests were carried out by applying puffs of humidified air (200 ml/min) flowing for 1 s using a stimulus controller through a Pasteur pipette containing a small piece of filter paper (Whatman no. 1) strip (1 cm^2) with 1 μl of one of the test compounds flowing in a stainless steel delivery tube (1 cm diameter) with the outlet positioned at approximately 1 cm from the antenna. In each experiment, the antenna was first presented with an injection of the standard reference compound, hexane (HPLC grade, Sigma-Aldrich Co.), and then with injections of ammonia. Based on highly specific sensitivity of insect olfactory sensory neuron (OSNs) to several compounds related with decomposition of protein-containing organic materials, such as ammonia [65], standard of ammonia (25% v/v aqueous solution, Merck KGaA, Darmstadt, Germany) was selected as test odorant. Puffs of the tested compounds were applied at 1 min intervals at least 8 times on each antenna. Replicates were performed with different individuals ($n = 13$ for conventional and $n = 15$ for transgenic).

Thermolimit respirometry recordings

The technique consisted of measuring respiration rates and thermal tolerance of seven individuals of *C. rutilans cyanescens* fed with pork feces with conventional maize and five individuals of *C. rutilans cyanescens* fed with pork feces with transgenic maize.

Dung beetles were individually placed in an analysis chamber of a thermal plate that would gradually increase the temperature. Each individual before being introduced into the analysis chamber was weighed on a precision balance. The temperature and behavior of each individual was controlled by infrared thermography using a FLIR ThermaCam P620 thermal infrared camera with a resolution of 640x480 pixels and a microbolometer Focal Plane Array detector with a spectral range of 7.5–13 mm and thermal sensitivity of 0.06ºC at 30ºC. The thermocamera was calibrated with the Standard Calibration service provided by FLIR Systems Inc. In each infrared image an alive and a death beetle (control) were used. For temperature measurement, body temperatures (thorax as reference) were taken over time using ThermaCAM^{TM} Researcher v 2.9 software.

Metabolic rates were estimated from oxygen consumption and carbon dioxide production. The measurement of respiration rate of dung beetles was quantified by exhaled CO_2 through the respiratory spiracles. The analysis is carried out through an analyzer CO_2 / H_2O LICOR-7000 coupled to a system air flow control (QUBIT-SYSTEMS), a temperature controller and dew point (HR-300 Water Vapor Analyzer, SABLE SYSTEMS) and a data acquisition module (data acquisition interface UI2, SABLE SYSTEMS) which connects all the devices to the computer. During the experiment, air was pumped through a column ascarite/drierite that remove CO_2 and H_2O before entering the chamber containing an individual. The air flow was regulated at 100 ml min^{-1}. The duration of each experiment was about 1 hour, coinciding with the death period of the individual.

Using the ThermaCAM Researcher v2.9 program, the sequences obtained by the thermographic video were recorded and analyzed. Sable Systems Expedata analysis software was used to process $\dot{V}_{CO2}$ measurements. CO_2 production were recorded in parts per million and, after data were converted to ml min^{-1}. Data were then transported into Excel program.

The thermographic video was compared to respirometry recordings (VCO_2 production) and the stages: spasm (S) and death (D) were detected in the thermographic videos. Death as determined by the last time that any slight motion was observed. The first peak of VCO_2 increased was called "stress peak" and the second peak of VCO_2 increased "posmortal peak". The beginning of the first peak with VCO_2

increased exponentially was called "entry stress peak" and the beginning of the second peak was called "entry posmortal peak".

Phenoloxidase (PO) analysis

Hemolymph extraction - Twenty six individuals of *C. rutilans cyanescens* undergoing two treatments were pinned in pronotum (lower lateral), placed in an eppendorf and centrifuged at 4000 rpm for 30 seconds. After, the hemolymph obtained was frozen at -80°C until analysis. PO activity was compared undergoing two treatments. According to Verdú and collaborators (2013), who stablished a protocol adapted for dung beetles, aliquots of 10 µl of hemolymph of two treatments were diluted to 200 µl in 50 mM phosphate buffered saline (PBS, pH 7.4). The hemolymph was centrifuged for 5 min at 20.000 rpm at 5°C and used immediately. Subsequently, 50 µl of the hemolymph solution was mixed with 50 µl of L-DOPA (20 mM) and added to the wells of a microplate. The absorbance was measured using a SPECTROstar Nano microplate reader. The microplate was incubated at 25°C for 5 minutes and after it was selected phenoloxidase assay (absorbance 492 nm, 25°C incubation). PO activity was determined by the slope of the reaction (absorbance - time) during the linear phase of the reaction (4-22 minutes). PO activity is expressed as units per ml of hemolymph, where one unit is the amount of enzyme required to increase the absorbance by 0.001 units per minute.

With the data from antenal response (amplitude and recuperation time), respirometry parameters and PO activity an analysis of variance and GLM were conducted in R 3.0.1 software (R CORE TEAM, 2013).

Results

Olfactory response

The EAG responses obtained for *C. rutilans cyanescens* consisted of a variety of olfaction potential amplitudes, showing clear differences in electrophysiological potential between the ammonia and hexane (blank). However, the EAG response to the ammonia of *C. rutilans cyanescens* fed with transgenic (6.56 ± 1.78 mV; $n = 15$) was not different from the response to the *C. rutilans cyanescens* fed with conventional (6.21 ± 0.04 mV; $n = 13$) (F = 0.1934, $P = 0.6637$), and the recuperation time in the two treatments was not different as well (KW-H = 0.577, $P = 0.447$).

Respirometry thermolimit

Twelve individuals of *C. rutilans cyanescens* were used in the experiment, seven individuals fed with conventional and five individuals fed transgenic derived dung, with an average weight of 0.142±0.02 g and 0.168 ± 0.02 g, respectively. When the temperature reached 47.25±0.52°C and 47.45±0.70°C for conventional and transgenic, respectively, a spasm was detected and a few time later the beetles stopped moving. The temperature of death was 48.24±0.46°C and 48.08±0.34°C for conventional and transgenic, respectively.

During thermal tolerance experiments, beetle $V CO_2$ increased with temperature, followed by a $V CO_2$ decline, then increased again to form a second peak (Figure 1). Increased CO_2 activity was detected in the stress period (second peak) by infrared videos. Subsequently a spasm was detected with the extension of posterior legs and after the individual death was observed. The individuals of *C. rutilans cyanescens* that consumed feces with GM maize showed higher values of $V CO_2$ emission compared with individuals that consumed feces with conventional maize during the stress period (Table 1, Figure 1). However, there was no difference between the quantity of $V CO_2$ emitted in the two treatments in: entry posmortal peak (F = 2.77, P = 0.12), posmortal peak (F = 1.82, P = 0.20). A significant difference was found in the entry stress peak (F = 5.81, P = 0.03) and stress peak (F = 5.52, P = 0.04) (Table 1, Figure 1).

Table 1: Parameters of thermolimit respirometry of *C. rutilans cyanescens* in two treatments, Conv- fed pork feces with conventional maize and Trans- fed pork feces with transgenic maize. * Asterisks indicate significant differences between treatments (*P*<0.05).

Parameters	Treatments	
	Conv	Trans
Body mass (live) (mg)	0.142±0.020	0.168±0.028
Entry stress Peak $V CO_2$ (ml CO_2/min)	0.0016±0.0011	0.0046±0.0021*
Stress Peak $V CO_2$ (ml CO_2/min)	0.0117±0.0031	0.0245±0.0115*
Entry Postmortal Peak $V CO_2$ (ml CO_2/min)	0.0044±0.0022	0.0070±0.0021
Postmortal Peak (°C)	47.67±2.5	47.32±2.6

Table 1:(**Continuation**): Parameters of…..

Postmortal Peak V CO_2 (ml CO_2/min)	0.0087±0.0030	0.0125±0.0051
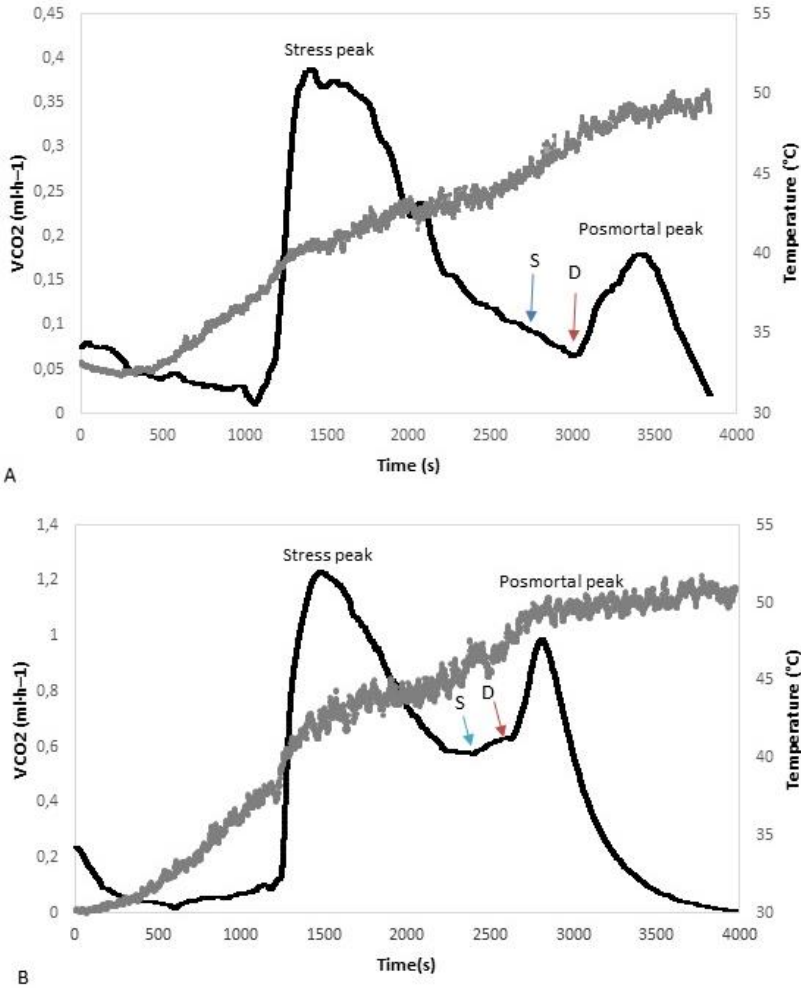
Figure 1: Values of body temperatures and $V$$CO_2$ emission with ambient temperature increment of two individuals of *C. rutilans cyanescens* sampled. (A) Conv, (B) Trans. S: spasm, D: death.

Phenoloxidase (PO)

The phenoloxidase (PO) reaction rate obtained for *C. rutilans cyanescens* was variable, showing differences in the two treatments. For the conventional diet, mean PO activity were 13.4 U/min/ml (minimum: 1.1 U/min/ml; maximum: 40.9 U/min/ml) and for dung beetles feeding transgenic, mean PO activity were 33.8 U/min/ml (minimum: 4.3 U/min/ml; maximum: 109.1 U/min/ml). The PO activity of *C. rutilans cyanescens* fed with transgenic (33.8±26.6; *n* = 13) was different from the response to the *C. rutilans cyanescens* fed with conventional (13.4±11.3; *n* = 13) (z value= 10.30; p=0.0002) (Figure 2).
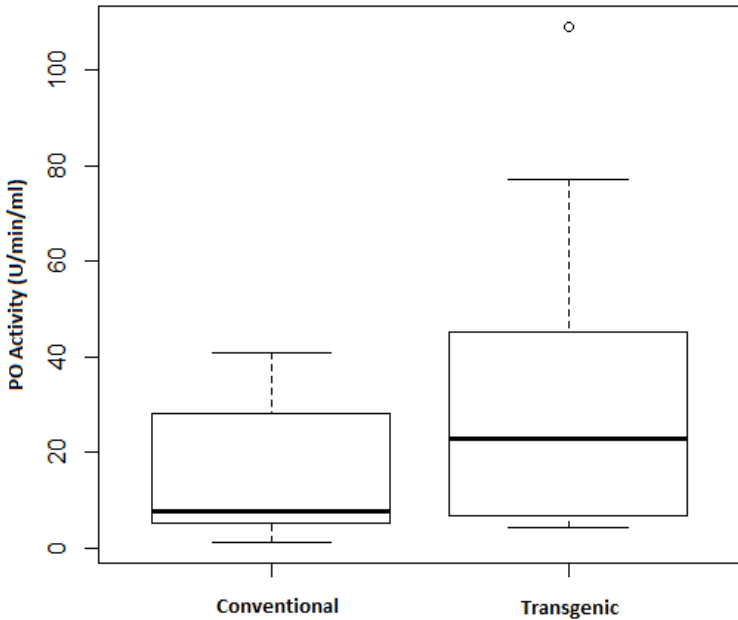
Figure 2: Phenoloxidase (PO) activity obtained for *C. rutilans cyanescens* in two treatments: fed with pork feces with conventional maize and with pork feces with transgenic maize.

Discussion

There was no difference in antennal response of *C. rutilans cyanescens* in the two treatments. The electroantennography results confirmed that the antennae of *C. rutilans cyanescens* respond to volatile compounds. However, we did not find any differences in the intensity of the EAG response of *C. rutilans cyanescens* in the two treatments. The transgenic maize (Bt) does not seem to affect this olfactory capacity differently from what occurs through the ivermectin, which affects the sensorial capacity of dung beetles (Verdú et al., 2015).

The maximum limit of thermal tolerance range of 47ºC to *C. rutilans cyanescens* in the two treatments showed that the maximum threshold temperature is close to the thermal shock (Verdú et al., 2006). *C. rutilans cyanescens* inhabit forest habitats and has low efficiency in heat dissipation. This characteristic of concentrating the heat in the thorax, makes limiting inhabit open areas, where sunlight coupled with endothermia would generate a lethal condition to the species.

In general, there was no difference of thermal tolerance and respiratory rate in the two treatments, but individuals of *C. rutilans cyanescens* that consumed feces with GM maize, showed higher values of CO_2 emission in the heat stress period. The differences found in this study between *C. rutilans cyanescens* in the two treatments were: (a) $V CO_2$ emission in the entry of stress peak and (b) $V CO_2$ emission in the stress peak.

It is known that various chemical (e.g. pesticides, drugs, metals), physical (e.g. radiation, temperature) and physiological (e.g. aging, disease) stressors can result in a stress situation that may upset functional homeostasis (Kodrík et al.*,* 2015). Stressed insects had a higher metabolic rate and anti-stress reactions are followed by enhancement of immune responses. Higher values of CO_2 exhaled during the respiration could indicate immune stress.

The present study suggests that occurs an increase in PO activity in response to a transgenic diet. The phenoloxidase (PO) activity was different, had an increase in the *C. rutilans cyanescens* individuals that consumed feces with transgenic. Thus, a diet based on feces with GM maize resulted in higher immune responses than a diet based on feces with conventional maize.

The complexity of the mechanism of action of *B. thuringiensis* has not been completely elucidated and the way of action of endotoxins is poorly understood (Ferre & Van Rie, 2002). However Bt resistance was

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associated with higher phenoloxidase activity in insects (Rahman et al., 2004; Ma et al., 2005), and possibly the increased phenoloxidase activity in dung beetles that consumed feces with transgenic is a response of immune system to infectious agents.

Environmental changes requiring special adaptation and Bt tolerance is associated with an elevated immune response (Rahman et al., 2004; Ma et al., 2005). A response of immune system is accompanied with high energetic cost and diminishes the insect's fitness and the cost of becoming a resistant insect against Bt toxins seem to be negative (Gassmann et al., 2009; Kliot & Ghanim, 2012). Stressful events or stress hormones may boost the immune response. This can be understood as a way of maintaining immune equilibrium in harmful environment (Adamo, 2012).

We propose that transgenic maybe acts as an environmental stressor to non-target organisms, such as dung beetles. Most published studies with transgenic were based on negative parameters such as mortality. More subtle effects, such as behavioral or physiological evidences, have not been studied to a comparable extent. Their importance, however, is evident.

Measurements for testing the existence of fitness costs performed in laboratory conditions can be useful for evaluating specific effects in dung beetles. More studies with longer exposure to a diet with transgenic and experiments with controlled transgenic protein concentrations are required for conclusive results.

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Supporting Information

S1 Table: EAG responses of indviduals of *C. rutilans cyanescens* in the two treatments. C: Conventional, T: Transgenic.

Treat-ment	Hexa-ne (mV)	Ammo-nia (mV)	Recupera-tion time (s)	Treat-ment	Hexa-ne (mV)	Ammo-nia (mV)	Recupera-tion time (s)
C1	0.17	3.64	9.8	T1	1.48	3.68	31.9
C2	0.56	7.48	24.0	T2	0.91	3.09	21.4
C3	1.22	7.77	16.38	T3	3.73	6.85	12.5
C4	0.33	4.5	16.0	T4	0.29	6.87	13.25
C5	0.39	7.9	21.58	T5	3.38	8.18	21.81
C6	0.04	7.7	18.63	T6	0.53	6.51	19.36
C7	0.51	7.55	22.21	T7	0.37	7.4	17.45
C8	0.15	3.1	12.6	T8	0.23	7.8	24.54
C9	0.61	7.86	22.75	T9	1.80	8.77	19.85
C10	0.62	7.87	22.00	T10	0.13	7.31	9.0
C11	0.39	7.55	14.41	T11	1.01	8.27	23.07
C12	0.18	4.9	18.55	T12	0.40	8.11	26.8
C13	0.26	2.97	11.37	T13	0.25	1.73	17.2
				T14	0.23	5.84	15.21
				T15	0.65	7.99	16.33

S2 Table: Values of CO_2 emissions with the environmental temperature increment for each individual of *C. rutilans cyanescens* sampled.

Dung beetles	CO_2 Stress Peak (ml·h–1)	CO_2 Post mortal Peak (ml·h–1)	CO_2 Entry Stress Peak (ml·h–1)	CO_2 Entry Posmortal Peak (ml·h–1)
C1	0.389	0.179	0.011	0.067
C2	0.369	0.452	0.026	0.159
C3	0.917	0.633	0.095	0.403
C4	0.507	0.691	0.030	0.231
C5	0.303	0.482	0.026	0.165
C6	0.553	0.566	0.136	0.391
C7	0.477	0.887	0.093	0.372
Mean	0.502	0.555	0.059	0.255
SD	0.134	0.158	0.041	0.114
T1	1.235	0.990	0.128	0.636
T2	0.803	0.368	0.195	0.317
T3	0.732	1.186	0.164	0.555
T4	1.637	0.618	0.108	0.510
T5	0.303	1.151	0.083	0.244
Mean	0.942	0.862	0.135	0.452
SD	0.395	0.295	0.034	0.137

Apêndice Capítulo 4: Fotografias dos procedimentos experimentais realizados na Universidade de Alicante/Espanha

Antena de escarabeíneo montada nos eletrodos, experimento de eletroantenografia.
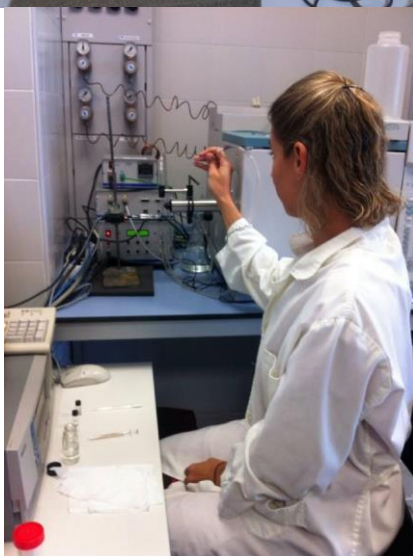
Pesquisadora aplicando sopros de ar dos compostos testados diretamente na antena do besouro.

Respirômetro com analisador de CO_2 e H_2O para medida de taxa metabólica de insetos.
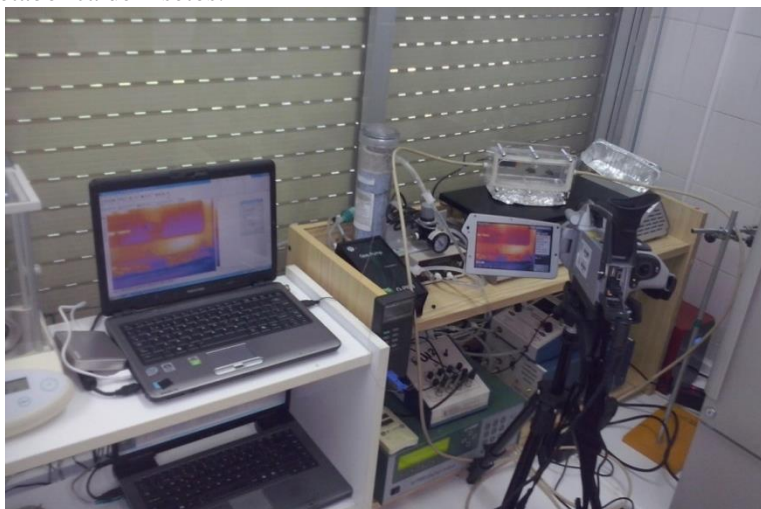
Termocâmera e respirômetro funcionando simultaneamente durante o experimento.

Exemplo de vídeo termográfico gerado com a termôcamera, besouro vivo à esquerda, morto à direita.

CONCLUSÕES GERAIS

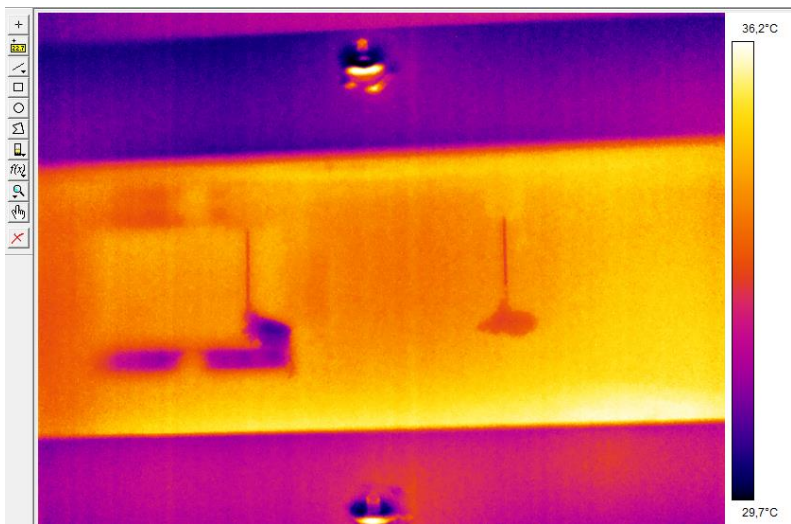Nossos resultados em campo mostraram que tanto a estrutura da comunidade de escarabeíneos quanto a composição são diferentes em fragmentos de Mata Atlântica cercados por milho transgênico, quando comparados com fragmentos cercados por milho convencional, confirmando estudos anteriores, onde foram detectadas diferenças na proporção de grupos funcionais e abundância de algumas espécies de escarabeíneos em fragmentos de Mata Atlântica cercadas por milho transgênico (CAMPOS & HERNÁNDEZ, 2015a). Além disso, as comunidades de escarabeíneos de fragmentos de Mata Atlântica perto de milho transgênico apresentaram menor riqueza de espécies, abundância e biomassa. Estas diferenças foram atribuídas ao tipo de milho (transgênico) e às técnicas de manejo de milho associadas a esse tipo de cultivo.

Além disso, as técnicas de manejo de milho associadas ao tipo de milho cultivado em torno dos fragmentos florestais influenciaram as comunidades de escarabeíneos presentes nos fragmentos. O efeito do manejo, especialmente as variáveis "presença de gado" e "uso de inseticidas", foram variáveis preditivas importantes para a comunidade de escarabeíneos. O uso de milho transgênico nessas áreas foi uma tentativa de diminuir a aplicação de inseticidas (embora em sete áreas com milho transgênico o inseticida foi aplicado), no entanto, a resposta da comunidade de escarabeíneos ao uso de transgênico foi pior em termos de riqueza e abundância de escarabeíneos do que em fragmentos adjacentes ao milho convencional que usaram inseticidas. A presença de gado e o uso indireto de ivermectina afetaram negativamente a composição e abundância dos besouros rola-bosta em fragmentos de Mata Atlântica cercados por milho. Os resíduos da ivermectina são liberados nas fezes, contaminam o ambiente e podem afetar os escarabeíneos (LUMARET & ERROUSSI, 2002; LUMARET & MARTÍNEZ, 2005; MARTÍNEZ & CRUZ 2009; VERDÚ et al., 2015).

A riqueza de espécies de besouros escarabeíneos ao nível da paisagem revelou uma grande diversidade, mesmo em uma região com muitos fragmentos de Mata Atlântica cercados por plantações de soja e milho (ver CAMPOS & HERNÁNDEZ, 2013; DA SILVA & HERNÁNDEZ, 2014). Pequenos fragmentos florestais são frequentemente referidos como habitats impróprios para muitos animais, incluindo mamíferos de grande porte e besouros coprófagos associados

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(ESTRADA et al., 1999). No entanto, neste estudo encontramos uma grande riqueza de mamíferos na região e, possivelmente, estes mamíferos usam esses pequenos fragmentos como trampolins ou corredores, para se deslocarem para grandes áreas. A distribuição dos escarabeíneos é fortemente influenciada pela diversidade de excrementos de mamíferos (DAVIS & SCHOLTZ, 2001; BOGONI & HERNÁNDEZ, 2014). Portanto, a diversidade de mamíferos na região poderia explicar a riqueza de espécies e abundância de besouros encontrados, uma vez que a estruturação da comunidade dos escarabeíneos é baseada na disponibilidade de recursos (*bottom-up*), e a competição espacial e temporal por recursos é um forte modificador da dinâmica populacional de escarabeíneos (HANSKI & CAMBEFORT, 1991).

Onze mamíferos e sete aves foram fotografados consumindo milho em fragmentos de Mata Atlântica e não houve preferência pelo tipo de milho quando o milho convencional ou transgênico foi oferecido. A diferença no consumo de milho nos diferentes fragmentos presumivelmente é devido à disponibilidade dos recursos presentes em cada fragmento e os consumos mais elevados, possivelmente, indicam poucos recursos disponíveis nesses fragmentos. O milho é um recurso alimentar abundante durante alguns períodos, e para os consumidores com dietas amplas - onívoros - um *trade-off* entre a disponibilidade de alimentos e a eficiência de forrageamento é necessária (FUTUYMA & MORENO, 1988). Em ambientes com altos níveis de degradação ambiental, há um número crescente de pássaros onívoros e, possivelmente, de aves insetívoras menos especializadas (MOTTA-JUNIOR, 1990), como observado neste estudo.

Pequenos mamíferos granívoros aumentam a abundância perto de plantações de milho, e campos de milho modificam a estrutura das comunidades de pequenos mamíferos tropicais, fornecendo um recurso complementar (BRAGA et al., 2015). Isto possivelmente muda toda a cadeia alimentar dentro destas paisagens, e mamíferos oportunistas de médio porte também podem ser beneficiados nessas áreas com o recurso complementar disponível (pequenos mamíferos e milho). Mamíferos oportunistas (por exemplo, *D. novemcinctus*, *C. thous, D. albiventris* e *N. nasua*) foram registrados na maioria dos fragmentos cercados por plantações, e os mamíferos generalistas foram as espécies mais fotografadas (por exemplo, *M. gouazoubira, D. novemcinctus* e *N. nasua*). Estas espécies são capazes de usar habitats transformados e

utilizar terras cultivadas perto das áreas florestais (ANDRADE-NÚÑEZ & AIDE, 2010; PINDER & LEEUWENBERG, 1997). Um estudo anterior na mesma região registrou pelo menos 21 mamíferos nativos usando fragmentos de Mata Atlântica cercados por plantações de milho (CAMPOS & HERNÁNDEZ, 2015b). Os efeitos ao longo da cadeia alimentar podem ser prejudiciais a estes mamíferos nativos, que podem sofrer com os efeitos tardios de culturas transgênicas, tais como os observados com ratos em laboratório (SÉRALINI et al., 2014). Se os transgênicos têm algum efeito tardio desconhecido em animais que se alimentam deste tipo de alimento, a fauna nativa também vai sofrer tais efeitos, uma vez que eles não fazem distinção entre os tipos de milho.

Efeitos negativos das culturas geneticamente modificadas ao longo da cadeia alimentar também podem estar ocorrendo, tais como mudanças nas comunidades de escarabeíneos (CAMPOS & HERNÁNDEZ, 2015a, 2015b), que usam as fezes de mamíferos como um recurso alimentar. Nas fezes de suínos alimentados com ração à base de milho transgênico foi encontrada a proteína Cry1Ab. A proteína já havia sido anteriormente detectada em fezes de mamíferos em outros estudos (CHOWDHURY et al., 2003; EINSPANIER et al., 2004; LUTZ et al., 2005; GUERTLER et al., 2008; PAUL et al., 2010) e a quantificação da proteína Cry1Ab foi semelhante à obtida em um estudo com suínos (CHOWDHURY et al., 2003).

Em laboratório, efeitos comportamentais foram detectados com besouros escarabeíneos alimentados com fezes de porcos que foram alimentados com ração à base de milho transgênico. O tempo para atingir o recurso (fezes) foi maior no tratamento transgênico e a quantidade de recurso enterrado foi maior no tratamento convencional. Além disso, detectou-se que escarabeíneos alimentados com transgênicos produziram mais bolas ninho. Os besouros rola-bosta competem por recursos alimentares escassos e efêmeros na natureza (HANSKI & CAMBEFORT, 1991; SIMMONS & RIDSDILL-SMITH, 2011) e se o tempo para alcançar o recurso alimentar for maior devido à alimentação indireta com transgênicos, esses indivíduos podem perder a competição para outros, diminuindo sua capacidade de chegar ao recurso. Consequentemente, podemos supor que as diferenças no "*fitness*" de escarabeíneos afetam as funções ecológicas desempenhadas por eles, tais como a remoção e enterrio de massas fecais (BRAGA et al., 2013). Escarabeíneos reduzem e incorporam massas fecais no solo, desempenhando um papel ecológico importante na ciclagem de

nutrientes com a decomposição da matéria orgânica e auxiliando na aeração do solo através de construção de túneis (NICHOLS et al., 2008), este comportamento de enterrio de recursos (fezes) foi menor nos besouros tuneleiros (*C. saphirinus*) quando tratados com alimento a base de milho transgênico.

Fêmeas de *C. rutilans cyanescens* alimentadas com fezes com transgênicos produziram mais bolas ninho, apesar do sucesso reprodutivo ter sido o mesmo em ambos os tratamentos. Besouros fêmeas investem uma grande quantidade de energia na construção de bolas ninho, as quais possuem um único ovo, e a variação ambiental e biológica pode resultar em diferentes estratégias reprodutivas (REANEY & KNELL, 2010). Um aumento do investimento na reprodução por causa do estresse ou mudanças no ambiente pode ocorrer a fim de maximizar o sucesso reprodutivo, sendo chamado de "investimento terminal" (CLUTTON-BROCK, 1984).

Muitos estudos ecotoxicológicos experimentais são feitos sobre reprodução e taxas de sobrevivência, mas não há registro do possível efeito indireto de transgênicos em escarabeíneos (organismo não-alvo), associados por meio da cadeia trófica. Somente em estudos de campo foram detectadas diferenças nas comunidades de escarabeíneos em fragmentos florestais cercados por milho transgênico (CAMPOS & HERNÁNDEZ, 2015a, 2015b) e estes experimentos suportam a ideia de que o milho GM estava afetando esta comunidade.

Nos experimentos ecofisiológicos, não houve diferença na resposta antenal de *C. rutilans cyanescens* quando comparados os dois tratamentos. Também não foram encontradas diferenças na intensidade da resposta eletroantenográficas de *C. rutilans cyanescens* nos dois tratamentos. O milho transgênico (Bt) não parece afetar essa capacidade olfativa diferentemente do que ocorre através da ivermectina, que afeta a capacidade sensorial dos escarabeíneos (VERDÚ et al., 2015). Em termos gerais, não houve diferença de tolerância térmica e respiratória nos dois tratamentos, mas os indivíduos de *C. rutilans cyanescens* que consumiram fezes com o milho GM, apresentaram maiores valores de emissão de CO_2 no período de estresse por calor. As diferenças encontradas neste estudo entre *C. rutilans cyanescens* nos dois tratamentos foram: (a) emissão de CO_2 na entrada do pico de estresse e (b) emissão de CO_2 no pico do estresse. Sabe-se que diversos estressores, químicos (por exemplo, pesticidas, drogas, metais), físicos (por exemplo radiação, temperatura) e fisiológicos (por exemplo,

envelhecimento, doença) podem conduzir a uma situação de stress que podem perturbar a homeostase funcional (KODRÍK et al., 2015). Insetos estressados tem uma taxa metabólica e reações anti-stress mais elevadas seguidas por aumento de respostas imunitárias. Os valores mais elevados de CO_2 exalado durante a respiração pode indicar estresse imunológico.

Os resultados também sugerem que ocorre um aumento na atividade de fenoloxidase (PO) dos escarabeíneos em resposta a uma dieta alimentar transgênica. A atividade da PO não foi significativamente diferente, mas ocorreu um aumento nos indivíduos de *C. rutilans cyanescens* que consumiram fezes com transgênicos. Assim, uma dieta baseada em fezes de porcos que se alimentaram com milho transgênico resultou em respostas imunitárias mais elevadas do que uma dieta baseada em fezes de porcos que se alimentaram com milho convencional.

A complexidade do mecanismo de ação de *B. thuringiensis* ainda não foi completamente elucidado e o modo de ação das endotoxinas é mal compreendido (FERRE & VAN RIE, 2002). No entanto a resistência a Bt já foi associada com maior atividade de fenoloxidase em insetos (RAHMAN et al., 2004; MA et al., 2005), e possivelmente o aumento de atividade de fenoloxidase nos escarabeídeos que consumiram fezes de porcos alimentados com transgênico é uma resposta do sistema imunológico a agentes infecciosos. Mudanças ambientais requerem uma adaptação especial e a tolerância a Bt está associada com uma resposta imunitária elevada (MA et al., 2005; RAHMAN et al., 2004). A resposta do sistema imunológico é acompanhada de alto custo energético e diminui a aptidão do inseto. O custo de se tornar um inseto resistente contra toxinas Bt parece ser negativo (GASSMANN et al., 2009; KLIOT & GHANIM, 2012). Eventos estressantes ou hormônios do estresse podem aumentar a resposta imunológica. Isto pode ser entendido como uma maneira de manter o equilíbrio imunológico em ambiente nocivo (ADAMO, 2012).

Propomos que o milho transgênico esteja agindo como um estressor ambiental para os organismos não-alvo (besouros escarabeíneos). O parecer de liberação do milho MON 810 pela CTNBIO, afirma que a proteína é tóxica somente para os insetos-alvo, especificamente para lepidópteros (lagartas) que possuem em seus intestinos receptores específicos para essa proteína, contudo a rota de exposição a organismos não-alvo, especificamente no caso de organismos detritívoros, não foi

devidamente estudada. A maioria dos estudos publicados com transgênicos foi negativo com base em parâmetros tais como a mortalidade, mas, efeitos sutis, tais como evidências comportamentais ou fisiológicas, não foram estudados em uma extensão comparável e a sua importância, no entanto, é evidente.

Efeitos não letais demonstrados pelo uso de transgênicos nesta pesquisa podem deixar os escarabeíneos menos competitivos pelos recursos efêmeros e aumentar a predação, resultando em populações diminuídas e, assim, explicar a perda da diversidade observada nas comunidades em fragmentos de floresta nativa (CAMPOS & HERNÁNDEZ, 2015b), o que pode resultar em uma diminuição dos serviços ecossistêmicos proporcionados por esses insetos.

Com base nos resultados obtidos no presente estudo e nas condições em que os trabalhos foram realizados é possível concluir que:

- As plantações de milho transgênico associadas ao manejo característico destas áreas influenciaram as comunidades de escarabeíneos presentes nos fragmentos de Mata Atlântica no sul do Brasil rodeados por estas plantações.
- As comunidades de escarabeíneos em fragmentos de Mata Atlântica rodeados de milho transgênico apresentam menor riqueza de espécies, abundância total e biomassa total.
- Dez espécies de mamíferos nativos e sete de aves foram fotografadas consumindo milho nos fragmentos de Mata Atlântica sem mostrar preferência de consumo por milho convencional ou transgênico.
- A proteína Cry1Ab passa através da cadeia trófica já que foi detectada nas fezes de porcos que se alimentaram com ração à base de milho transgênico e a concentração média de Cry1Ab nas fezes foi de 304±45,96 ng/g.
- A quantidade de fezes enterrada por indivíduos de *Coprophanaeus saphirinus* foi menor naqueles que se alimentaram com fezes de porcos alimentados com ração à base de milho transgênico quando comparadas com aqueles que consumiram fezes de porcos alimentados com ração à base de milho convencional.
- A incorporação de matéria orgânica por casais de *C. rutilans cyanescens*, assim como o sucesso reprodutivo, foi similar nos besouros que se alimentaram com fezes de porcos que se alimentaram com ração à base de milho transgênico comparados

aos que se alimentaram com fezes de porcos alimentados com ração à base de milho convencional.

- Os escarabeíneos que se alimentaram indiretamente com transgênico produziram mais bolas ninhos, embora o sucesso reprodutivo (número de indivíduos que emergiram) foi similar nos dois tratamentos após três meses de experimento.
- Em um experimento de olfatometria, os indivíduos de *Canthon rutilans cyanescens* que se alimentaram com fezes de porcos que se alimentaram com ração à base de milho transgênico levaram mais tempo para chegar ao recurso.
- Em um experimento de eletroantenografia, nenhuma diferença foi detectada nas respostas antenais dos escarabeíneos que se alimentaram com fezes de porcos provenientes de milho transgênico e convencional.
- Nos experimentos de respirometria e atividade de fenoloxidase foram detectadas evidências de aumento da atividade do sistema imune: aumento da emissão de CO_2 durante a respiração e aumento da atividade de fenoloxidase nos indivíduos de *Canthon rutilans cyanescens* que se alimentaram com fezes de porcos que se alimentaram com ração à base de milho transgênico.

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