Inbreeding and measures of immune function in the cricket *Teleogryllus commodus*

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Studies of sexual selection and immunity in invertebrates often assay components of the immune system (e.g., encapsulation response, hemocyte counts) to estimate disease resistance. Because increased disease resistance is thought to enhance fitness in most cases, we might expect a positive relationship between fitness and measured immune function. Indeed, several studies have shown that measures of immunity are correlated with fitness enhancing traits. We used inbreeding to investigate the relationship between fitness and 2 commonly used assays of insect immunity in the cricket *Teleogryllus commodus*. Previous studies in *T. commodus* have shown inbreeding depression for several life history and sexually selected traits. We compared the lysozyme-like activity of the hemolymph and hemocyte counts of inbred (full-sibling mating) and outbred crickets. If these measures of immune function are positively correlated with fitness, we expect both measures to decline with inbreeding. However, there was no change in lysozyme-like activity and a significant increase in hemocyte counts with inbreeding. Our results demonstrate that it is not always the fittest individuals that have highest measured immune function. Key words: fitness, hemocytes, immunity, inbreeding, lysozyme, sexual selection, *Teleogryllus commodus*. [Behav Ecol 22:486–492 (2011)]
yet underexploited method of creating classes of individuals that can be assigned high or low fitness is to manipulate fitness using inbreeding (e.g., Bolund et al. 2010; review: Cotton et al. 2004). Inbreeding increases homozygosity and usually results in a decline in fitness known as inbreeding depression (review: Keller and Waller 2002; Armbruster and Reed 2005). This reduction in fitness mainly arises due to the unmasking of partially or fully recessive deleterious alleles as well as the loss of advantageous heterozygosity (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009). Inbreeding could affect the immune system due to lower heterozygosity at loci that directly affect traits involved in immunity or by reducing the general ability of individuals to acquire and/or assimilate the energetic and nutrient resources that are available to be allocated to the immune system (i.e., lowering condition sensu Rowe and Houle 1996; review: Tomkins et al. 2004). More generally, inbreeding should reduce many components of fitness, such as disease resistance, performance capacity, and condition, and therefore, inbred individuals can be unambiguously assigned as low fitness relative to outbred individuals. In this study, we investigated the relationship between 2 widely used measures of immune function and fitness by comparing the immune function of inbred and outbred individuals. We investigated the effect of one generation of full-sibling mating on lysozyme-like activity in the hemolymph and hemocyte counts in Teleogryllus commodus, a gryllid cricket endemic to Australia. Lysozyme is an antibacterial enzyme that hydrolyses bacterial cell walls. Its activity is assayed by measuring the rate at which an individual’s hemolymph clears a bacterial suspension (e.g., Rantala and Roff 2005). Hemocytes are the cellular components of hemolymph, and a subset of these cells are involved in an immune response by transporting molecules to the site of infection, by ingesting foreign particles or by encapsulating parasites (Korner and Schmid-Hempel 2004; Łomniczak et al. 2006). Inbreeding reduces fitness across a wide range of taxa (Crnokrak and Roff 1999; Keller and Waller 2002; Armbruster and Reed 2005) and has strong negative effects in T. commodus. One generation of inbreeding (full-sibling mating) in T. commodus causes a reduction in hatching success, nymph survival, and adult lifespan and affects sexually selected male traits, such as calling effort and call structure (Drayton et al. 2007, 2010). We therefore assume that inbred crickets are less fit and in poorer condition (sensu Rowe and Houle 1996) than their outbred counterparts. Both hemocyte counts and lysozyme-like activity have been used as a proxy for immunity and disease resistance (e.g., Rolff 2001; Ahitaiinen et al. 2004; Rantala and Kortet 2004; Simmons and Roberts 2005; Simmons et al. 2005; Tregenza et al. 2006) and have been shown to correlate with fitness enhancing traits (e.g., Ryder and Siva-Jothy 2000; Ahitaiinen et al. 2004, 2006; Rantala and Kortet 2004) in other invertebrate species. If, in T. commodus, either of these measures are correlated with individual condition or fitness, we expect both of these measures to decline with inbreeding. Empirical tests directly testing whether less fit individuals show reduced expression of specific immune components are rare. Such tests are therefore needed.

MATERIALS AND METHODS

Generation and rearing of inbred and outbred crickets

Inbred individuals were created by full-sibling matings (inbreeding coefficient, $F = 0.25$), and their fitness compared with that of outbred crickets ($F = 0$ assuming panmixis in the field population, which is in an area surrounded by many square kilometers of suitable habitat). Full-sibling families were derived from ~70 wild-caught gravid females collected in May 2006, at Smith Lakes, NSW, Australia. To start, the offspring of these wild-caught females were reared to adulthood and separated before sexual maturity to ensure virginity. They were then paired with a non-sibling (i.e., different wild-caught mother) to create full-sibling families. Each full-sibling family was reared communally in a $43 \times 30 \times 13$-cm plastic tub with dry cat food (KiteKat Krunch; Uncle Ben’s, Raglan, Australia) and water ad libitum. Females were separated from their brothers as soon as their ovipositors became visible to ensure virginity.

We created 33 experimental blocks that each used 2 unreared full-sibling families (i.e., not derived from the same wild-caught female). In each block, brothers and sisters from both full-sibling families were mated to create 2 inbred genotypes. Outbred genotypes were created by reciprocal mating of a male and a female from each family (see Figure 1). This design generated 4 offspring genotypes (2 inbred and 2 outbred) per block. It was balanced for the relative maternal and paternal genetic contribution of each family to inbred and outbred offspring, which is necessary to control for variation among families in breeding values for focal traits (see Tomkins et al. 2010). After mating females were provided with moist cotton wool (“egg pads”) in which to lay eggs. We checked egg pads every 3 days for newly hatched nymphs. Nymphs from the same mother were reared communally for 20 days in $9 \times 9 \times 5$-cm plastic tubes with food and a piece of moist cotton wool. They were then transferred to individual containers ($9 \times 9 \times 5$ cm) with a vial of water plugged with cotton wool, cat food, and a cardboard egg cup for shelter. Nymphs were not transferred to individual containers immediately after hatching to reduce the risk of being crushed by their water tube. In total, we set up 5073 nymphs. The density during the communal stage was very similar because, on average, we set up $37.5 \pm 3.5$ ($\pm$ standard error [SE]) nymphs of each inbred genotype and $39.4 \pm 3.3$ nymphs of each outbred genotype per block. Food and water were replaced every 10 days. As nymphs neared maturity, we checked daily for the molt into adulthood to record their development time (i.e., days taken to reach maturity). All adults used to test the effect of inbreeding on immune function were $\geq 10$ days post maturation to ensure that they were sexually mature. Furthermore, because crickets were reared and maintained in individual containers (apart from the brief period of communal rearing as nymphs), all crickets used in the experiment were virgins. Crickets were maintained at 26–28 °C on a 12:12 photoperiod.

Lysozyme-like activity in the hemolymph

We used a sterile pin to make a small puncture under the pronotum from which we collected 2 μl of hemolymph with a Gilson pipette. This was added to 8 μl of phosphate-buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 in 1 l distilled water, pH 7.4) and frozen to induce

<table>
<thead>
<tr>
<th>Block</th>
<th>Matings</th>
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<tbody>
<tr>
<td>1</td>
<td>A♂ A♀</td>
</tr>
<tr>
<td>2</td>
<td>C♂ C♀</td>
</tr>
<tr>
<td>3</td>
<td>E♂ E♀</td>
</tr>
<tr>
<td>4</td>
<td>G♂ G♀</td>
</tr>
<tr>
<td>5</td>
<td>H♂ H♀</td>
</tr>
</tbody>
</table>

Figure 1

The mating design showing Block 1 (comprising full-sibling families A and B), Block 2 (comprising full-sibling families C and D), and so on. There are 2 inbred and 2 outbred offspring genotypes per block.
cell lysis. The samples were later thawed and 90 µl of a *Micrococcus lysodeikticus* solution (3 mg/ml PBS) added. We then loaded samples into a microplate spectrophotometer (PowerWave 340; Bio-Tek Instruments Inc., Winooski, VT) to measure lysozyme activity at 490 nm and 30 °C as the rate of change in optical density from 10 to 30 min and from 0 to 80 min. These 2 measures were highly correlated ($r = 0.991$, $P < 0.001$, $n = 1015$), and we only present analyses using the later. Crickets were weighed before hemolymph extraction (mass and linear body size, such as pronotum width, are closely correlated, $r = 0.946$, $P < 0.001$, $n = 2731$; Jennions M, unpublished data). Crickets were 18–25 days post maturation (22.2 ± 0.06 days) when hemolymph was taken. There were no age differences between inbred males, inbred females, outbred males, and outbred females (Kruskal–Wallis test: $\chi^2 = 0.587$, $P = 0.899$). Lysozyme activity measured in this way is highly repeatable ($r = 0.74$, $P < 0.001$, Drayton J, Boeke J, Jennions M, unpublished data).

Hemolymph samples from all groups (inbred males, inbred females, outbred males, and outbred females) were run together on the same MicroWell plates (Nunc, Roskilde, Denmark). Several control samples (containing PBS and *M. lysodeikticus* solution, but no hemolymph) were also loaded onto each plate to control for any daily fluctuations in the assays. The rate of change in optical density for each hemolymph sample was then calculated as the sample slope minus the control slope. This was then multiplied by −1 to make the value more intuitive (i.e., a greater value indicates more lysozyme-like activity in the hemolymph).

### Hemocyte counts

At the same time that we collected hemolymph for the lysozyme assays, an additional 2 µl of hemolymph was collected and mixed with 8 µl of anticoagulant (3.92 g NaOH, 8.532 g NaCl, 6.328 g ethylenediaminetetraacetic acid, 8.616 g citric acid in 1 l distilled water). This mixture was then expelled onto a Neubauer hemocytometer, and we counted the hemocytes in 6 of the 0.04-mm² central squares, following a checker-board pattern, to estimate the number of circulating hemocytes per milliliter of hemolymph. For 56 crickets, we counted the hemocytes in a further 6 squares to measure the repeatability of the count. It was repeatable ($F_{30.56} = 3.132, P < 0.001, r_I = 0.521$).

### Statistics

To test for an effect of inbreeding on lysozyme-like activity and hemocyte counts, we ran separate linear mixed models estimated using restricted maximum likelihood in S + 7.0. Where necessary, variables were transformed to ensure that residuals were normally distributed and homoscedastic. We used a model simplification approach, initially fitting a full model, including all 2-way interactions and then removed fixed terms until the final model only contained significant terms (random terms were always retained) (Crawley 2002). First, we tested for an effect of inbreeding on lysozyme activity and hemocyte counts. Inbreeding was included as a fixed factor in the models. Sex and adult age (days post maturation) were included as a fixed factor and a fixed effect covariate, respectively. Block and the interaction between inbreeding and block were included as random factors. This controlled for variation in the mean value of immune parameters across blocks (i.e., random intercepts) and differences in the effect of inbreeding among blocks (i.e., random slopes). We then added the 2 life-history traits (development time and weight at hemolymph collection) to the models as fixed effect covariates to investigate any relationships between immune parameters and life-history traits. To allow the reader to assess the influence of terms excluded from the final models, we present the significance value associated with the parameter estimate for each term if it is included in the final model. We tested the repeatability of our hemocyte counts using a one-way analysis of variance with cricket identity as the factor. Unless otherwise stated summary statistics are mean ± SE, tests are 2-tailed and $\alpha = 0.05$. To allow comparison of the biological importance of different variables, we calculated standardized effect sizes by converting the $F$-statistic for each model parameter into the common currency of Pearson’s $r$ using the MetaWin 2.0 calculator (Rosenberg et al. 2000).

### RESULTS

#### Lysozyme-like activity in the hemolymph

Summary statistics are presented in Table 1. Lysozyme-like activity was measured for 452 inbred (204 males, 248 females) and 578 outbred crickets (237 males, 341 females). The relationship between age at hemolymph collection and lysozyme-like activity differed significantly between the sexes ($F_{1,906} = 11.178, P = 0.001$), so we ran separate models for each sex.

Inbreeding had no effect on the lysozyme-like activity of males or females. Older females had higher lysozyme-like activity levels than younger females, but there was no effect of age for males. For males, the relationship between development time and lysozyme-like activity differed between inbred and outbred crickets ($F_{1,382} = 7.054, P = 0.008$), so to assess

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The effect of 4 variables on lysozyme-like activity in the hemolymph</th>
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<tbody>
<tr>
<td></td>
<td>Lysozyme-like activity of males</td>
</tr>
<tr>
<td></td>
<td>df</td>
</tr>
<tr>
<td>Inbreeding</td>
<td>1.25</td>
</tr>
<tr>
<td>Age at hemolymph collection</td>
<td>1.383</td>
</tr>
<tr>
<td>Development time</td>
<td>Inbred: 1.173</td>
</tr>
<tr>
<td>Weight at hemolymph collection</td>
<td>Inbred: 1.174</td>
</tr>
</tbody>
</table>

$P$ values are from the final model if the term was significant or when it alone was added to the final model if nonsignificant (see text). $P$ values significant at the 0.05 level are in bold.

The effect size $r$ is $+/−$ to show the direction of the change in lysozyme-like activity with inbreeding ($+ =$ increase with inbreeding) or the direction of the correlation with the 5 continuous traits, df, degrees of freedom.
the effect of life-history traits on lysozyme-like activity, we ran separate models for inbred and outbred males. Outbred males who took longer to reach sexual maturity had higher lysozyme-like activity, but there was no such relationship for inbred males. Heavier inbred males had lower lysozyme-like activity, but there was no such relationship for outbred males. The relationship between weight and lysozyme activity did not differ significantly between inbred and outbred males \((F_{1,380} = 3.468, P = 0.063, \text{although the interaction was marginally nonsignificant})\). There was no relationship between female lysozyme activity and either development time or weight (Table 1).

**Hemocyte counts**

Summary statistics are presented in Table 2. Hemocyte counts were made for 329 inbred (159 males, 170 females) and 369 outbred crickets (179 males, 190 females). Inbreeding significantly increased the number of circulating hemocytes, and females had significantly more hemocytes than males (Figure 2). Crickets that took longer to mature had higher hemocyte counts. There was no detectable effect of adult age or body mass at the time of hemolymph collection on hemocyte count (Table 2).

**Relationship between hemocyte count and lysozyme-like activity**

When we included lysozyme activity in the final model for hemocyte counts, we found that crickets with higher lysozyme activity also had more circulating hemocytes in their hemolymph \((F_{1,503} = 215.52, P < 0.001, r = 0.5477)\) (Figure 3). This relationship did not differ between the sexes \((F_{1,502} = 0.000, P = 0.997)\) or between inbred and outbred crickets \((F_{1,502} = 0.684, P = 0.409)\).

**DISCUSSION**

Studies of vertebrates suggest that inbreeding can reduce immune defense and increase susceptibility to parasitism and disease (e.g., Townsend et al. 2009; reviewed in Keller and Waller 2002). However, studies with nondomesticated vertebrates often rely on indirect measures of inbreeding. For example, they compare individuals with varying levels of heterozygosity, or those from small and large populations, rather than performing controlled breeding experiments (but see Ilmonen et al. 2008 and references within for exceptions). Furthermore, without such experiments, it is difficult to control for the level of inbreeding in the parents of inbred individuals, thereby potentially confounding estimates of inbreeding depression in offspring (e.g., Szulkin and Sheldon 2006). Invertebrates are more amenable to controlled breeding designs, but studies investigating inbreeding depression in invertebrate immunity have reported mixed results. One generation of brother–sister mating significantly reduced the encapsulation response of female, but not male, pupae of the moth *Epirrita autumnata* (Rantala and Roff 2007) and had no effect on encapsulation ability in the bumblebee *Bombus*

**Table 2**

<table>
<thead>
<tr>
<th>Hemocyte counts</th>
<th>df</th>
<th>F</th>
<th>P</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inbreeding</td>
<td>1.27</td>
<td>5.912</td>
<td><strong>0.022</strong></td>
<td>+0.4238</td>
</tr>
<tr>
<td>Sex</td>
<td>1.637</td>
<td>23.148</td>
<td>&lt;0.001</td>
<td>0.1873</td>
</tr>
<tr>
<td>Age at hemolymph collection</td>
<td>1.636</td>
<td>2.664</td>
<td>0.103</td>
<td>+0.0646</td>
</tr>
<tr>
<td>Development time</td>
<td>1.637</td>
<td>6.692</td>
<td><strong>0.010</strong></td>
<td>+0.1029</td>
</tr>
<tr>
<td>Weight at hemolymph collection</td>
<td>1.636</td>
<td>3.319</td>
<td>0.069</td>
<td>+0.0721</td>
</tr>
</tbody>
</table>

*P* values are from the final model if the term was significant or when it was added to the final model if nonsignificant (see text). *P* values significant at the 0.05 level are in bold.

The effect size *r* is +/− to show the direction of the change in hemocyte counts with inbreeding (+ = increase with inbreeding) or the direction of the correlation with the 3 continuous traits, df, degrees of freedom.
terrestris (Gerloff et al. 2003). Inbred and outbred termites, Zootermopsis angusticollis do not differ in their encapsulation response, or in survival following bacterial and fungal infections when kept individually. When kept in groups and exposed to high doses of fungal disease, however, inbred termites had reduced survival and higher cuticular microbial loads. This suggests that inbreeding does not affect the immunity of individuals but might affect social mechanisms of pathogen control, such as allogrooming (Calleri et al. 2006).

Inbred lines of the red flour beetle, Tribolium castaneum, did not differ from the original outbred stock population in their susceptibility to tapeworm infection (Stevens et al. 1997). Crickets, Gryllus firmus, from inbred lines did not differ from individuals resulting from “outbred” crosses between lines in encapsulation ability or the lysozyme-like activity of the hemolymph (Rantalainen et al. 2007). In contrast, however, decorated crickets Gryllodes sigillatus from inbred lines had a greater encapsulation response than outbred crickets (Gershman et al. 2010). Such differences between studies may reflect differences in the genetic architecture of immune parameters between species, populations, or even between the sexes. For example, in the abovementioned study of the moth E. autunnata, the author’s findings suggest that in males, genetic variation for the encapsulation response is predominantly additive. In females, however, additive variation is reduced, and encapsulation shows higher levels of directional dominance (Rantalainen and Roff 2007). Because inbreeding depression is due to the effects of directional dominance (Lynch and Walsh 1998), as expected, inbreeding depression was found in female, but not male, encapsulation response (Rantalainen and Roff 2007).

Our results using the cricket T. commodus do not support the prediction that inbreeding reduces two widely used measures of immunity. Instead, inbreeding was associated with a significant increase in hemocyte count, and there was no effect on lysozyme-like activity. There are several possible explanations for the higher number of circulating hemocytes in inbred crickets. First, if inbred T. commodus are more susceptible to pathogens, they might upregulate their immune defense to fight current infections. Challenges to the immune system are known to increase measures of immune function, including hemocyte counts, in several insects (e.g., Moret and Schmid-Hempel 2000; Ahmed et al. 2002; Korner and Schmid-Hempel 2004). It is therefore possible that the increased hemocyte counts of inbred crickets is a response to increased levels of infection. However, challenges to the immune system should also increase lysozyme-like activity in crickets (see Jacot et al. 2005), and we did not find any increase in the lysozyme-like activity of inbred individuals. Second, if inbred crickets have a reduced investment in other fitness traits, they might, as a byproduct, invest more into hemocyte production than outbred crickets. For example, in a study with similar results to our own, Gershman et al. (2010) found that inbreeding increased encapsulation ability in decorated crickets, Gryllus sigillatus. The authors speculated that because inbred crickets have reduced reproductive effort, they have more resources to invest into immunity. How inbreeding affects egg laying or provisioning in female T. commodus is unclear, but inbred males do have reduced calling behavior (Drayton et al. 2010). Third, inbreeding might disrupt physiological mechanisms responsible for regulating hemocyte production resulting in overproduction. If so, inbred crickets risk an overly responsive immune system, which could result in autoimmunity (e.g., Sadd and Siva-Jothy 2006) and/or the economical use of energetic or nutritional resources diverted to immunity (e.g., Siva-Jothy and Skarstein 1998; Lochmiller and Deerenberg 2000). Given the increase in the number of circulating hemocytes with inbreeding, it seems plausible that the function relating hemocyte count to fitness is nonlinear, so that there is an optimal peak, where counts well below this optimum run the risk of severe pathology, and counts above carry autoimmune and resource costs (Siva-Jothy and Skarstein 1998).

Inbreeding in normally outbreeding species almost always reduces offspring fitness (Falconer and Mackay 1996) and has strong negative effects on key life history and sexually selected traits in T. commodus (Drayton et al. 2007, 2010). We are confident that the inbred crickets used in the current study suffered comparable reductions in fitness to those reported in the previous studies of Drayton et al. (2007, 2010) for the following reasons. First, although they were a separate group of crickets, the inbred crickets used in the current study were bred (i.e., full-sibling mating) and reared under almost identical conditions to those used by Drayton et al. (2007). Second, the crickets used by Drayton et al. (2010) (where substantial inbreeding depression was found in male calling behavior) are the same individuals used in the current study to investigate the effect of inbreeding on immunity. We can therefore be confident that our inbred crickets have reduced fitness. Both hemocyte counts and lysozyme-like activity have been used as a proxy for disease resistance in other vertebrates (e.g., Rolff 2001; Ahtiainen et al. 2004; Rantalainen and Kortet 2004; Simmons and Roberts 2005; Simmons et al. 2005; Tregenza et al. 2006), and increased disease resistance is thought to enhance fitness in most cases (but see Viney et al. 2005). Furthermore, hemocyte count and lysozyme-like activity have been shown to correlate with fitness enhancing traits (e.g., Ryder and Siva-Jothy 2000; Ahtiainen et al. 2004, 2006; Rantalainen and Kortet 2004). If lysozyme-like activity and hemocyte counts correlate positively with fitness in T. commodus, we would expect both to decline with inbreeding. For any of the reasons listed above, however, we have shown that inbred individuals had a larger hemocyte count (and therefore potentially higher immunocompetence) than outbred individuals. Furthermore, there was no difference in lysozyme-like activity between inbred and outbred crickets. Our results demonstrate that it is not always the fittest individuals that have highest measured immune function.

It is worth noting that there are 2 general approaches to measuring immune function, either by measuring baseline levels (prechallenge or constitutive: e.g., Simmons and Roberts 2005; Simmons et al. 2005; Tregenza et al. 2006) or by measuring levels after an immune challenge is administered (induced: e.g., Adamo 2004a; Gershman et al. 2010). Our study investigated the effect of inbreeding on constitutive immunity. Although it would have been informative to also investigate the effect of inbreeding on induced immunity, we had to balance the pursuit of large sample sizes needed for a statistically powerful test of inbreeding effects (our sample sizes are far larger than those of most comparable studies) against the logistical constraints of measuring both constitutive and induced immunity. There is, however, evidence that constitutive and induced immune measures are correlated in Telogryllus. Specifically, prechallenge hemocyte counts correlate with the degree of encapsulation of a nylon filament insert in both T. oceanicus and T. commodus (Simmons and Roberts 2005; Simmons et al. 2005; Tregenza et al. 2006). The relationship between pre and postchallenge lysozyme-like activity in T. commodus is currently unknown. It is plausible, however, that baseline levels reflect immunocompetence as a high baseline level of lysozyme could facilitate a more rapid immune response against invading pathogens. Furthermore, in scorpionflies Panorpa vulgaris, baseline lysozyme-like activity is positively correlated with the phagocytic capacity of the hemolymph, which is an important defense against particles, such as bacteria and fungal spores (Kurtz et al. 2000). In Gryllu.
Inbreeding depression, however, although the increase in lysozyme-like activity in response to an immune challenge predicted survival after bacterial infection, baseline lysozyme activity level did not (Adamo 2004a). Whether this is a general finding that will be replicated in other species is unknown.

In conclusion, in *T. commodus*, 2 commonly used measures of immune function were not correlated with fitness if we make the reasonable assumption that inbreeding lowers fitness. One generation of inbreeding increased one measure of immune function and did not affect another. Our empirically robust results are based on very large sample sizes and involved a direct experimental manipulation of fitness. They provide empirical support for more general warnings of equating greater immunity with elevated fitness (e.g., Viney et al. 2005).

**CONFLICT OF INTEREST**

None declared.

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