1 *Maternal androgens in avian brood parasites and their hosts:*
2 *responses to parasitism and competition?*

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19 ABSTRACT

20 In the coevolutionary dynamic of avian brood parasites and their hosts, maternal (or
21 transgenerational) effects have rarely been investigated. We examined the potential role of elevated
22 yolk testosterone in eggs of the principal brood parasite in North America, the brown-headed
23 cowbird, and three of its frequent host species. Elevated maternal androgens in eggs are a common
24 maternal effect observed in many avian species when breeding conditions are unfavorable. These
25 steroids accelerate embryo development, shorten incubation period, increase nestling growth rate,
26 and enhance begging vigor, all traits that can increase the survival of offspring. We hypothesized
27 that elevated maternal androgens in host eggs are a defense against brood parasitism. Our second
28 hypothesis was that elevated maternal androgens in cowbird eggs are a defense against intra-
29 specific competition. For host species, we found that elevated yolk testosterone was correlated with
30 parasitized nests of small species, those whose nest success is most reduced by cowbird parasitism.
31 For cowbirds, we found that elevated yolk testosterone was correlated with eggs in multiply-
32 parasitized nests, which indicate intra-specific competition for nests due to high cowbird density.

We propose experimental work to further examine the use of maternal effects by cowbirds and their hosts.

Keywords: arms race; cowbird; maternal effects; parasitism; tolerance; yolk testosterone

1. Introduction

Maternal effects occur widely in plants and animals, but the role of maternal effects has been little examined in obligate brood parasites and their hosts. Maternal effects are reproductive investments that modify the phenotype of progeny in ways that increase a female's reproductive success under changing environmental conditions (Mousseau and Fox 1998). In avian species, it is widely recognized that elevated yolk hormones are one of the principal maternal effects provided to offspring (Price 1998, Sockman, Sharp et al. 2006, Gil 2008, Bentz, Navara et al. 2013, Duckworth, Belloni et al. 2015). The literature on elevated yolk androgens has shown that both physiological and behavioral traits are altered, including accelerated embryo development, shortened incubation period, accelerated nestling growth, and more vigorous begging behavior (Schwabl 1993, Reed and Vleck 2001, Strasser and Schwabl 2004, Daisley, Bromundt et al. 2005, Groothuis, Eising et al. 2006). These altered traits provide a competitive advantage to female birds whose offspring hatch under unfavorable environmental conditions such as limited food supply, high population density, or predation pressure (Schwabl 1993, Schwabl, Mock et al. 1997, Bonneaud, Mazuc et al. 2003, Pilz and Smith 2004, Gil 2008).

Parasitic birds do not build nests or provide parental care. Instead they exploit other species by laying eggs in their nests and usurping their parental care, usually reducing the breeding success of the host species (Ortega 1998, Rothstein and Robinson 1998, Davies 2000). In North America, the principal brood parasite is the brown-headed cowbird (*Molothrus ater*), a host-generalist that exploits over 200 different host species (Lowther 1993, Ortega 1998). Smaller species experience disproportionately greater reduction in fledging success, both because the cowbird egg can disrupt incubation of small host eggs due to its larger size and shorter incubation period and also because cowbird nestlings can dominate access to food due to aggressive behavior in obtaining parental care and larger size (Friedmann 1929, Briskie and Sealy 1990, Lowther 1993, Lichtenstein and Sealy 1998, Rothstein and Robinson 1998).

1.1. Host defenses and elevated maternal testosterone

We hypothesized that elevated maternal androgens function as a host defense when cowbird parasitism is a threat. A variety of defenses against cowbird parasitism have been studied in songbird species, including nest abandonment, egg ejection, burying the egg under nest material, and physically chasing female cowbirds away from the nest (Friedmann 1929, Lowther 1993, Sealy 1996, Rothstein and Robinson 1998, Sealy, Neudorf et al. 1998, Klippenstine and Sealy 2008, Pagnucco, Zanette et al. 2008, Guigueno and Sealy 2010). Effective nest concealment and minimal activity around the nest during host egg-laying have also been reported to be associated with lower levels of parasitism (Nice 1937, Ortega 1998, McMaster, Gill et al. 2000, McLaren, Woolfenden et al. 2003).

We hypothesized that elevated yolk androgens provide a defense against cowbird parasitism by altering offspring phenotype in ways that make them more competitive in obtaining parental care. Not only do elevated yolk androgens produce offspring that grow faster and beg more aggressively, they can also increase hatching success of host young. Host survival is critically dependent on the timing of host egg hatching relative to that of the cowbird (Norris 1947, Berger 1951). Accelerated embryo development shortens the host incubation period, increasing the probability that at least some host young will hatch close to the hatch time of the cowbird and that their age and size disadvantage will be reduced.

We predicted that elevated yolk testosterone characterizes the eggs of host species in nests that have been parasitized. We investigated three songbird species that are frequently exploited by cowbirds, all smaller than the cowbird and representing a range of body sizes. The smallest species, American redstart (*Setophaga ruticilla*), is one-quarter the size of the female cowbird (Sherry and Holmes 1997); the largest, veery (*Catharus fuscescens*), is close in size to the female cowbird (Norris 1947); and the red-eyed vireo (*Vireo olivaceus*), is intermediate in size between the other two species (Cimprich, Moore et al. 2000)(Table 1). We tested our prediction by collecting eggs from both parasitized and unparasitized nests, then comparing yolk testosterone concentration of the eggs.

1.2. Cowbird competition and elevated maternal testosterone

Our second hypothesis concerned the occurrence of maternal effects in the brood parasite itself. Elevated yolk androgens are associated with high population density and increased intra-specific competition in a number of avian species, including house sparrows (Schwabl, Mock et al. 1997), barn swallows (Whittingham and Schwabl 2002), tree swallows (Bentz, Navara et al. 2013),

and European starlings (Pilz and Smith 2004). We hypothesized that elevated yolk androgens in cowbirds would also be associated with high population density, because that creates more intra-specific competition for host nests (Donovan 1994, Rivers, Jensen et al. 2010, Rivers, S. Young et al. 2012).

One indication of high cowbird population density is the presence of multiply-parasitized nests (Smith and Arcese 1994, Hahn, Sedgwick et al. 1999, Smith, Cook et al. 2000, Trine 2000, McLaren, Woolfenden et al. 2003). When host nests are plentiful, cowbirds typically avoid laying more than one egg in a host nest (Hann 1937, Norris 1947, Elliot 1977, Smith and Arcese 1994, Ellison, Sealy et al. 2006, Rivers, Jensen et al. 2010). However, when the number of breeding females is large relative to the available number of host nests, they begin to lay more than one egg in a host nest.

Cowbird hatching and fledging success is lower in multiply-parasitized nests. Trine (2000) showed that each additional cowbird egg in a wood thrush nest reduced cowbird hatching success by 8 – 10%. Similarly, Smith and Arcese (1994) showed that cowbird fledging success was 20% lower in multiply-parasitized nests than in singly-parasitized nests. A second cowbird egg in a nest means increased competition for both young cowbirds, and hatching and fledging success of cowbirds are both lower in multiply-parasitized nests. By dispersing her eggs among different host nests, a female cowbird (i) reduces competition among her offspring; (ii) reduces the risk of nest abandonment, since host birds desert a nest more frequently when there is more than one cowbird egg (Hann 1937, Norris 1947, Berger 1951); and (iii) spreads the risk of loss among several nests, since songbird nests fail frequently due to predators, weather, poor nest site or nest construction ((Mayfield 1965, Ehrlich, Dobkin et al. 1988).

The tendency of cowbirds to avoid laying more than one egg in a nest has been determined by field studies using genetic tools to identify the maternity of each egg (Alderson, Gibbs et al. 1999, Strausberger and Ashley 2003, Woolfenden, Gibbs et al. 2003, Rivers, S. Young et al. 2012). For example, only one of 15 multiply-parasitized nests contained eggs from the same female at our study site in an earlier study (Hahn et al. 1999). At other sites, the proportion of multiply-parasitized nests with eggs from the same female was higher, particularly in prairie habitats and landscapes with extensive agriculture and abundant cowbirds, but the proportion is usually below 50% (Alderson, Gibbs et al. 1999, Strausberger and Ashley 2003, Woolfenden, Gibbs et al. 2003, Rivers, S. Young et al. 2012).

We hypothesized that cowbird eggs in multiply-parasitized nests are characterized by elevated yolk testosterone, since the competitive traits produced by this maternal effect are advantageous for young cowbirds just as they are for young host nestlings. Consequently we compared the yolk testosterone level of cowbird eggs in multiply-parasitized nests to that of cowbird eggs in singly-parasitized nests. Our study sample of cowbird eggs was obtained by collecting eggs as they were laid in the host nests we were monitoring (*see Section 1.1*).

2. Materials and methods

2.1 Study species

We studied three host species that are frequent targets of cowbird parasitism (Ehrlich, Dobkin et al. 1988, Terborgh 1989, Hahn and Hatfield 1995, Johnsgard 1997). All three species are smaller than the cowbird, and they represent a range of body sizes from one-quarter the size of the cowbird (American redstart), to one-third the size (red-eyed vireo), to close to the size of the cowbird (veery). A side-by-side comparison of species size differences is presented in Table 1.

American Redstart The redstart is a New World warbler (Parulidae) with a broad geographic range (Sherry and Holmes 1997, Marra, Hobson et al. 1998). Redstarts favor interior woodland over edges and prefer large tracts of habitat. They build their nests at mid-level heights from 0.5 - 2 m in dense bushes or at the fork formed by a branch and the trunk; clutch size is 3-5. The American redstart is one of the species that brown-headed cowbirds parasitize most frequently throughout their range, and redstarts experience severely reduced reproductive success when parasitized (Friedmann 1963, Sherry and Holmes 1997). Both males and female redstarts respond aggressively to cowbirds in their territory (Ficken and Ficken 1962).

Impact of cowbird parasitism: Cowbird parasitism causes a very severe reduction in redstart success: Powell and Knudsen (2006) reported that 31% of parasitized redstart nests raised only the cowbird (14 / 45) and 62% raised a partial clutch of redstarts as well as a cowbird.

Red-eyed Vireo The red-eyed vireo (Vireonidae) winters in the Amazon basin and has a large breeding range extends across most of North America except the southwest (Cimprich, Moore et al. 2000). Nests are in deciduous trees at heights of 3 – 6 m high, and the clutch is typically 4 eggs. This vireo is one of the songbirds parasitized most frequently by cowbirds in the woodlands of eastern North America (Cimprich, Moore et al. 2000).

Impact of cowbird parasitism: Cowbird parasitism severely reduces the reproductive success of red-eyed vireos (Donovan, Thompson et al. 1995). Southern (1958) reports that, when parasitized, only 38% - 48% of vireo nests fledged one or more vireo chicks.

Veery Veery is a medium-sized thrush (Turdidae) (Moskoff 2005) that is an area-sensitive breeder (i.e. density increases as patch sizes increases)(Donovan and R.H. Lamberson 2001) and prefers forests of at least 20 hectares. Veery breeds in early successional, damp, deciduous forest. It builds its nests on or near the ground, rarely higher than 6m hi, and it lays a clutch of 3-5 eggs (Ambuel and Temple 1983). Veery is frequently parasitized by brown-headed cowbirds (Hahn and Fleischer 1995, Burke and Nol 2000, Hames, Rosenberg et al. 2002, Falk, Nol et al. 2011).

Impact of cowbird parasitism: Veery experiences a low to moderate reduction in fledging success when its nests are parasitized (Schmidt and Whelan 1999). Norris (1947) reported that parasitized nests reduced fledging success from 2.5 veeries per unparasitized nest to 2.0 veeries per nest.

Brown-headed Cowbird This cowbird is a medium-sized songbird in the family of New World blackbirds (Icteridae) (Lowther 1993, Jaramillo and Burke 1999), and it has a broad geographic distribution covering most of the North American continent (Rothstein 1994, Rothstein and Robinson 1998, Sauer, Hines et al. 2005). The genus *Molothrus* is comprised of five parasitic species, the only avian brood parasites in the New World, and the brown-headed cowbird is the principal brood parasite in North America (Johnsgard 1997, Davies 2000).

An obligate brood parasite, the cowbird never builds its own nest or provides parental care, but exploits other species to serve as foster parents that raise its young. These foster parents incubate the cowbird eggs and feed young cowbirds from hatching till independence approximately six weeks later (Hahn and Fleischer 1995). Unlike the European cuckoo, which is a host-specialist, the cowbird is a host-generalist parasite and exploits over 200 species across its range (Lowther 1993, Ortega 1998). In an avian community where cowbirds are present, most of the songbird species in the community are parasitized (Lowther 1993, Rothstein and Robinson 1998).

Cowbird parasitism reduces nest success of most host species, although smaller host species typically experience greater reduction in nest success than larger species (Ortega 1998). Smaller egg size and smaller nestling size relative to the size of the cowbird egg and nestling create disadvantages that are associated with lower host hatching success and lower host fledging success.

Cowbirds typically lay a single egg in a host nest, but as cowbird population density increases relative to the number of host nests, the incidence of multiple parasitism increases (Ellison, Sealy et al. 2006, Rivers, S. Young et al. 2012). Cowbirds have lower hatching and fledging success in multiply-parasitized nests (Smith and Arcese 1994, Trine 2000).

Cowbird density is influenced by food availability, and cowbirds are more abundant in landscapes with livestock and agriculture (Robinson, Hoover et al. 2000). Multiple parasitism is more frequent in such areas (Trine 2000, McLaren, Woolfenden et al. 2003, Strausberger and Ashley 2003, Rivers, Jensen et al. 2010).

2.2 Study Site: Nest searching and egg collection

We worked in a 1300 hectare oak-maple-hemlock forest, with stands ranging in age from 70-150 years, on land belonging to the Rockefeller University Field Research Center, Millbrook, NY (Dutchess County) in 2001. The surrounding landscape is a mosaic of pasture, livestock, and suburban development that supports a large cowbird population (Dufty and Wingfield 1986, Glitzenstein, Canham et al. 1990, Hahn and Hatfield 1995, Schmidt and Whelan 1999, Schmidt 2001). The avian community has a rich diversity of songbird species, 83% of which are parasitized by cowbirds (n = 24 species)(Hahn and Hatfield 1995).

Nests of the three study species were located by searching systematically along transects within a 400 hectare block between April 1 and June 30. The study included only eggs from nests found before incubation had begun, since incubation initiates metabolism and could interfere with accurate measurement of the yolk testosterone deposited by the female. Once we located a nest under construction, we monitored it daily and collected each egg on the day it was laid. For each nest, the entire host clutch was collected. If a cowbird egg was laid, that was also collected.

We replaced each egg that we collected with an artificial egg in order to eliminate cues that can cause nest desertion. Once collected, eggs were taken to the laboratory, yolk and albumen were separated, and yolks were frozen and stored at -90 degrees C until assays were carried out.

2.3 Radio immunoassay

We measured yolk testosterone in three assays. The androgen assay followed modified protocols (Wingfield and Farner 1975, Wingfield, Hegner et al. 1991, Schwabl 1993). When laboratory analysis was begun, we weighed individual thawed egg yolks and homogenized them with 3 mL of distilled water. We took approximately 180 μ L of this homogenate to use in the

hormone assay, added 20 μ L of tritiated steroid hormone (for calculation of extraction recoveries), and equilibrated overnight. The next morning we added 300 μ L distilled H₂O, and extracted hormones twice with 4 mL diethyl ether. The combined extractions were dried under nitrogen, re-suspended in 1 mL of 90% ethanol and frozen overnight. Next, we centrifuged the sample for 5 min at 1,500 rpm to remove precipitated proteins and lipids. The remaining ethanol phase was decanted and again dried down under nitrogen. The dried extract was reconstituted with 0.5 mLs of 10% ethyl acetate in iso-octane. Reconstituted samples were then transferred to diatomaceous earth columns mixed with propylene and ethylene glycols as the stationary phase. Separation of androgens was achieved by eluting fractions in increasing concentrations of ethyl acetate in iso-octane (Wingfield and Farner 1975). Radioimmunoassay followed the protocols of Wingfield and Farner (1975) and Wingfield, Hegner et al. (1991). Intra-assay variability was 10%, calculated as the coefficient of variation from an assay of 10 duplicate standards by BGW. The choice to measure only testosterone was determined because testosterone is the major androgen, and during the breeding season, nothing substantive is added by measuring other androgens (Gil 2008)(Wingfield, pers. obs.).

2.4 Statistical Methods

2.4.1 Host eggs: assessing maternal effects in eggs in parasitized nests

To assess whether testosterone is elevated in eggs in parasitized nests, we compared the median testosterone level of eggs in parasitized nests to that of eggs in unparasitized nests using a non-parametric test since the data were not distributed normally (Siegel 1956, Miller 1998). We used a one-tailed test, because our hypothesis was directional and because the literature reports only elevated yolk T, not depressed yolk T, in response to particular environmental conditions (Gil 2008). We used “nest” as the experimental unit and calculated the average yolk T of eggs within a nest, then used this estimate to adjust the variance.

2.4.1.2 Goodness of Fit tests

In avian studies, it is conventional to use “nest” as the experimental unit when investigating differences among eggs in different nests, but “egg” can be used if circumstances warrant and Goodness of Fit tests (GoF) indicate that eggs within nests are not more highly correlated than eggs in different nests (Dziak, Coffman et al. 2012). We used Goodness-of-Fit statistics from repeated

measures analyses to assess independence for each host species. Two models were examined: the first used the data for each egg and assumed independence among eggs. The second fit a repeated measures model, computing the correlation among eggs within a nest. The Bayesian Information Criterion (BIC) was used to assess the fit of the two models (Appendix, Table 1).

For one of the host species, American redstart, the assumption of independence was accepted, and the GoF analysis supported use of either “nest” or “egg”. Consequently, we carried out both comparisons and presented the results of both analyses (Table 2).

For another host species, red-eyed vireo, the two models were only marginally different in fit. The GoF analysis yielded a result in the range (0 - 2) where there is no consensus about cut-off value. Consequently, we carried out two analyses for the vireo as we did for the redstart, and we presented the results from both analyses (Table 2)

For the third host species, veery, the eggs within a nest were highly correlated. The GoF analysis did not support the use of “egg” as the experimental unit. Consequently, only the analysis using “nest” was carried out. The results of the analysis using “nest” are presented in Table 2.

2.4.2. Cowbird eggs: assessing maternal effects in multiply-parasitized nests

To assess the hypothesis that cowbird eggs laid in multiply-parasitized nests are characterized by elevated yolk testosterone, we used a non-parametric test since the data were not distributed normally (Siegel 1956, Miller 1998). We carried out a one-tailed test, because our hypothesis was directional and because there is no biological basis to expect lower yolk testosterone levels in the experimental nests. The literature on maternal effects reports instances when elevated yolk testosterone is associated with particular environmental variables, but reports no instance when yolk testosterone level was depressed below its normal range (Gil 2008).

We carried out the comparison of yolk T in cowbird eggs in singly vs. multiply-parasitized nests using “nest” as the unit of analysis and the median yolk T values of eggs within each nest.

We also conducted the analysis using “egg” as the unit of analysis. The results of both are shown in Table 3. Together these two analyses represent the best estimate available when no genetic data is available on the genetic relatedness of different cowbird eggs (*see Section 2.4.2.1 below*).

2.4.2.1 The parasitic breeding system creates uncertainty about relatedness of cowbird eggs

In the case of parasitic birds, the convention to use “nest” to compare eggs in different nests should be evaluated to determine if it is appropriate. For example, cowbird females do not place all their eggs in a single nest as non-parasitic birds do. Instead, they disperse their eggs among many different nests (Ortega 1998, Rothstein and Robinson 1998, Davies 2000). Cowbirds typically lay their eggs in different nests within a defined home-range or territory (Dufty 1982, Darley 1983, Hahn, Sedgwick et al. 1999).

2.4.2.1.1 Singly-parasitized nests Cowbird eggs collected from different host nests may have been laid by the same female, especially if the nests lie within a particular female cowbird’s territory or home range (Hahn, Sedgwick et al. 1999). Accordingly, although the eggs are found in different host nests, they should be analyzed in a way that controls for correlations among the eggs. At the same time, cowbird eggs collected from different nests may have been laid by different cowbirds, and it is not appropriate to control for correlations among such eggs. Genetic data on the maternity of each cowbird egg is needed to know when to use individual egg values and when to take the median value of related eggs.

2.4.2.1.2 Multiply-parasitized nests Field studies using genetic data have shown that most often a multiply-parasitized nest contains eggs laid by different cowbirds, but sometimes contains eggs laid by the same female (Alderson et al. 1999; Hahn et al. 1999; Strausberger and Ashley 2003; Woolfenden et al. 2003; Rivers et al. 2010). The proportion of multiply-parasitized nests in which the eggs were laid by one cowbird varies among communities from a reported low of 7% (Hahn, Sedgwick et al. 1999) to a reported high of 55.7% (Rivers, Jensen et al. 2010).

When the eggs in a multiply-parasitized nest were laid by different cowbirds, the individual values of each egg should be used in the analysis. When the eggs were laid by the same cowbird, the median value should be used for the nest. The spatial issue of cowbird territories applies to both multiply-parasitized nests and singly-parasitized nests: if several multiply-parasitized nests lie within a cowbird female’s territory, she may lay an egg in more than one multiply-parasitized nest. As was the case for singly-parasitized nests, the analysis should control for eggs laid by the same female when they are laid in different multiply-parasitized nests.

2.4.2.1.3 Appropriate analysis in the absence of genetic data

In sum, the nature of the cowbird’s parasitic breeding system creates uncertainty about relatedness of cowbird eggs that must be considered in a comparison of yolk T level between cowbird eggs in singly-parasitized vs. multiply-parasitized nests. With genetic data that identifies the female who laid each egg, the comparison could be made by treating eggs as independent data

points when they are laid by different females and by taking the median of related eggs that were laid by the same female. However, in the absence of genetic data, the best estimate of the difference is provided by using both analyses (“egg” and “nest”), because the results of the two analyses will bracket the true p-value.

2.4.3 Seasonal change and level of yolk testosterone

Systematic changes in yolk T across the breeding season could influence the comparison of host eggs in parasitized and unparasitized nests or could be an alternate explanation of the patterns we observed. Consequently, we analyzed the relationship between lay date and concentration of yolk testosterone for each species, using Spearman rank correlation.

2.4.4. Host identity and level of yolk testosterone

If yolk T level of cowbird eggs were significantly different in the nests of different host species, that could influence our comparison of cowbird eggs in singly- and multiply-parasitized nests, or it could be an alternate explanation of the patterns we observed. Consequently, we compared the level of yolk testosterone of cowbird eggs in nests of the three host species using Kruskal-Wallis.

3. Results

3.1 Host defenses and elevated maternal testosterone

3.1.1 Smallest and largest host species

American redstart The redstart was the smallest host species we studied, and its eggs exhibited a significant elevation of yolk T in parasitized nests. Yolk T concentration of eggs in parasitized nests was significantly higher than that of eggs in unparasitized nest in the analysis using “nest” as the experimental unit ($P < 0.014$) (Table 2, section 1A). The difference was also significant in the analysis using “egg” as the experimental unit ($P < 0.041$) (Table 2, section 1B).

Veery Veery is the largest host species we studied and the only one whose eggs did not show elevated yolk T in eggs in parasitized nests (Table 2, section 3).

3.1.2 Intermediate-sized host: red-eyed vireo

Eggs of red-eyed vireo in parasitized nests showed a trend toward elevated yolk T, but the results were equivocal. Using “egg” as the experimental unit, an approach supported by the

Goodness-of-Fit statistic for red-eyed vireo (*see Appendix, Table 1*), we found that yolk T of vireo eggs in parasitized nests was elevated significantly compared to vireo eggs in unparasitized nests (4.8 ng/g in unparasitized nests vs. 7.3 ng/g in parasitized nests; $P < 0.009$)(Table 2, section 2A). However, the elevation of yolk T of eggs in parasitized nests was not significant when “nest” was used as the experimental unit (5.5 ng/g in unparasitized nests vs. 8.8 ng/g in parasitized nests; $P = 0.083$) (Table 2, section 2B).

3.2 Cowbird competition and elevated maternal testosterone

In multiply-parasitized nests, the yolk T level of cowbird eggs showed a strong trend toward elevated yolk T. Yolk T of cowbird eggs was significantly higher in multiply-parasitized nests than in singly-parasitized nests when “egg” was used as the unit of analysis ($P < 0.024$)(Table 3A). The elevation of yolk testosterone in eggs in multiply-parasitized nests was not significant when “nest” was used as the unit of analysis, ($P < 0.063$)(Table 3B).

The applicability of the “nest” analysis must be evaluated in the context of the cowbird’s parasitic breeding system (*as discussed in section 4.2.1*). “Egg” should be used appropriate for those eggs laid in host nests by different cowbirds, whether they were laid in singly-parasitized or multiply-parasitized nests, while “nest” is appropriate only for eggs laid by the same cowbird, whether they were laid as single eggs in different host nests or laid together in a multiply-parasitized nest.

In the absence of genetic data, neither analysis is sufficient alone. Since there is uncertainty about relatedness of different cowbird eggs (*see Section 2.4.2.1*), the best estimate of the difference in yolk T level of cowbird eggs in singly- vs. multiply-parasitized nests is given by the range of p-values determined by the two analyses presented ($0.024 < P < 0.063$)(Table 3).

3.3 Seasonal change and yolk testosterone concentration

The Spearman rank correlation using egg lay date and testosterone concentration was not significant for any of the species: American redstart ($r_s = 0.08$, $P = 0.653$); red-eyed vireo ($r_s = -0.12$, $P = 0.498$); veery ($r_s = -0.04$; $P < 0.825$); and cowbird ($r_s = 0.21$, $P = 0.34$).

3.4 Host species identity and concentration of yolk testosterone in cowbird eggs

A comparison of testosterone levels of cowbird eggs laid in nests of different host species found no difference among species (Kruskal Walls 3.04; $P < 0.218$).

4. Discussion

4.1. Mechanism for depositing elevated yolk androgens

The mechanism for most avian maternal effects is not known (Price 1998, Tschirren, Saladin et al. 2005, Groothuis and Schwabl 2008). However, the duration and sequence of events involved in cowbird parasitism allow sufficient time for additional yolk T to be deposited in eggs of both host and cowbird in response to environmental cues. Cues that trigger a mechanism to deposit extra yolk T must be perceived more than 24 hours before the egg is laid, because androgens accumulate in the egg only until the time the egg arrives in the oviduct where it is shelled. After 20-24 hours in the oviduct, the egg is laid (Groothuis and Schwabl 2008). For both host birds and cowbirds, there are pertinent environmental cues that could stimulate a trigger mechanism.

Pertinent cues for host species During nest building, host birds frequently receive cues that their nest is threatened by cowbird parasitism. For example, it is well established that female cowbirds frequently perch and intently watch a host pair nest-building (Friedmann 1929, Hahn, Sedgwick et al. 1999, Banks and Martin 2001). The duration and intensity of such host-watching has been noted so frequently that investigators have suggested that it may be an important stimulus for cowbird egg-laying (e.g. Berger 1951). It has also been reported frequently that host species recognize cowbirds as a threat and that nest owners respond aggressively and give alarm calls when cowbirds approach during nest-building (Robertson and Norman 1976, Smith, Arcese et al. 1984, Lowther 1985, Burghman and Picman 1989, Neudorf and Sealy 1992, Banks and Martin 2001, Smith, Taitt et al. 2002, Gill and Sealy 2004).

Since a host pair spends several days building a nest, then several days laying a clutch, one egg per day, the cue of a cowbird watching or approaching during nest building occur in time to trigger deposition of extra yolk testosterone. Cowbird egg are most often laid on the same day or the day after the first host egg (Berger 1951), so this cue occurs in time to trigger deposition of extra yolk T in host eggs.

Pertinent cues for the brood parasite For cowbirds, pertinent cues that intraspecific competition is high include (i) observation of the number of cowbirds in local feeding or roosting flocks and (ii) the degree of difficulty of finding a host nest at the right stage that does not already have a cowbird egg. Cowbirds perceive these cues as soon as they begin searching for nests.

Cowbirds have a longer time frame than do host species to perceive pertinent cues and deposit additional yolk T, because they do not experience the full sequence of breeding behaviors and underlying hormones (Dufty and Wingfield 1986). Specifically, cowbirds do not shift from nest-building to egg laying to incubation to parental feeding, and consequently cowbirds do not have the accompanying sequence hormone changes that underlie these behaviors (Wingfield 2005). For cowbirds, the breeding season is simply an extended, multi-week period of egg-laying.

4.2 Maternal effects as a host defense

This is the first report of maternal androgens potentially serving as a host defense against cowbird parasitism, and it suggests that the phenomenon merits further investigation, both experiments and field observations. Elevated yolk testosterone is one of the most common maternal effects employed by birds in response to a variety of unfavorable environmental conditions (Price 1998, Sockman, Sharp et al. 2006, Gil 2008). Cowbird parasitism has a negative impact on host breeding success analogous to poor food supply, high population density, and high predation rate (Berger 1951, Ortega 1998, Rothstein and Robinson 1998, Smith, Cook et al. 2000). Elevated yolk testosterone would produce an alternate host offspring phenotype with traits that increase the probability of survival when a nest has been parasitized.

We found significantly elevated maternal testosterone in eggs of the smallest host species studied, American redstart, in parasitized nests, but this pattern did not characterize the largest host species we studied. The redstart is approximately one-quarter the size of the cowbird, and its reproductive success is greatly reduced by nest parasitism. In contrast, the largest species, veery, showed no evidence of elevated yolk T in eggs in parasitized nests. Veery is close in size to the cowbird, and its nest success is only moderately reduced by nest parasitism.

Notably, the results for the red-eyed vireo were equivocal. Red-eyed vireo is intermediate in size between American redstart and veery (Table 1), and the degree of elevation of yolk T in its eggs in parasitized nests was also intermediate that of redstart and veery (Table 2).

Balancing costs and benefits of elevated yolk testosterone: a role for host size? Elevated yolk androgens have significant costs in addition to the benefits for embryos and nestlings. These include negative effects on: (1) immunity (Berger 1951, Ortega 1998, Rothstein and Robinson 1998, Smith, Cook et al. 2000); (2) growth (Pitala, S. Ruuskanen et al. 2009); (3) survival (Muller, Groothuis et al. 2005); (3) lifespan; and (4) contrasting positive and negative effects on male and

female nestlings (Sockman, Schwabl et al. 2001, Pitala, S. Ruuskanen et al. 2009, Ruuskanen, Doligez et al. 2012). The use of elevated yolk testosterone thus represents an evolutionary trade-off between costs and benefits.

Our results suggest that host body size may influence the cost/benefit calculation for this maternal effect. Very small species like the American redstart experience such severe reduction in reproductive success when cowbirds parasitize their nests that the benefits of more offspring surviving to fledge may outweigh the costs of such as shorter lifespan. In contrast, for larger species like veery that experience only moderate reduction in reproductive success due to cowbird parasitism, the benefits of elevated yolk T may not outweigh the costs. Our findings that larger host species do not have elevated yolk T in parasitized nests are consistent with the findings of Royle et al. (2011) who analyzed eggs of red-winged blackbird and found that yolk T was not elevated in parasitized nests (*Agelaius phoeniceus*).

The equivocal degree of elevation of yolk T in red-eyed vireo eggs suggests that the cost-benefit calculation for this maternal effect may be close in the vireo. If vireo size were close to a threshold that determined whether this maternal effect should be used, then elevation of yolk T in parasitized nests might be inconsistent among vireo females. Future work could fruitfully examine whether host size relative to cowbird size plays a role in triggering this maternal effect by examining eggs in parasitized nests of host species of different sizes.

Tolerance: a novel defense against avian brood parasitism This study provides the first evidence of a songbird using a tolerance defense against cowbird parasitism. Tolerance defenses are those that limit the severity of a parasite's impact without reducing the intensity of parasitism (Bush, Lafferty et al. 1997, Raberg, Sim et al. 2007). Traditionally, studies of host defenses against cowbird parasitism have identified only *resistance* defenses, those that reduce the intensity of brood parasitism such as nest guarding, physical aggression toward cowbirds, abandonment of nests that have been parasitized, nest-building over a cowbird egg, puncturing, burying, or ejecting cowbird eggs (Rothstein 1977, Sealy 1996, Davis and Sealy 2000, Smith, Cook et al. 2000, Peer, Rothstein et al. 2005),

The discovery of a potential tolerance defense against cowbird parasitism broadens our understanding of cowbird - host dynamics and encourages further exploration on the use of tolerance defenses. We suggest they may be more characteristic of small host species for whom

resistance defenses such as physically chasing off cowbirds are less effective (Raberg, Sim et al. 2007).

Within-species comparisons Our study and Royle et al.'s (2011) study of red-winged blackbird demonstrate the use of within-species comparisons in investigating associations between environmental and social factors and the level of maternal androgens in yolk. Both studies compared eggs of single species in parasitized nests vs unparasitized nests. We also presented a within-group comparison of cowbird eggs in singly-parasitized nests vs. multiply-parasitized nests.

Two early studies of maternal effects measured yolk androgens in the brown-headed cowbird and several host species (Hauber and Pilz 2003, Hahn, Hatfield et al. 2005) at a time before a consensus had been reached that yolk hormone levels are species-specific, determined by phylogeny, and that comparison of yolk androgen levels *among* species is not a valid way to show that an environmental factor is associated with this maternal effect (Gil 2008). Yolk androgens were measured in common cuckoo (*Cuculus canorus*) and compared to yolk androgens of its host, the great reed warbler (*Acrocephalus arundinaceus*) (Hargitai, Csaba et al. 2010), but it cannot be assumed that the relative sensitivity of hormone receptors will be the same in different species.

4.3 Competition among cowbirds: a role for maternal effects

Our results support the hypothesis that cowbirds, like non-parasitic birds, respond to increased population density and intra-specific competition with maternal effects. Since host nests are a principal focus of intra-specific competition among cowbird females and since multiply-parasitized nests are associated with high population density, cowbird eggs in multiply-parasitized nests are strong candidates to exhibit maternal effects (Table 3).

We suggest that it would be fruitful to explore whether *inexperienced* breeders deposit higher levels of yolk T in eggs compared to *experienced* cowbird females. McLaren, Wooldfendon et al. (2003) suggested that first year breeders contributed disproportionately to multiply-parasitized nests in Ontario, and a mechanism to explain this phenomenon would be that inexperienced cowbirds have a lower threshold for depositing yolk T in their eggs. Inexperienced cowbirds are likely to find nests more slowly -- they arrive at the breeding grounds without knowing the landscape, not having a territory or memory of the location of host nests the previous year (Wingfield 2005) -- and they must learn to find host nests at the right stage in the laying cycle (Berger 1951), so they are at a disadvantage competing with experienced females to find host nests

in which to lay their eggs. When cowbird density is high, we predict that inexperienced females are more likely to lay the 2nd (or 3rd) egg in a parasitized nest and to utilize maternal effects that increase the competitive traits of their offspring.

4.4 Future work: experimental manipulation studies

Experimental manipulation is a valuable way of establishing causation and moving beyond the correlative results presented here. It is important to be aware that experimental manipulation studies of cowbird parasitism are difficult to simulate, so it may be judicious to combine experiments with field observations of natural experiments, such as we present here.

Host eggs in parasitized nests: To establish causation for maternal effects in host eggs, experimental manipulations can be done with cues that are perceived as threats of cowbird parasitism such as: (1) insertion of a real or model cowbird egg in a host nest after the 1st host egg has been laid; (2) presentation of a stuffed or model female cowbird near a host nest during nest-building; and (3) playback of cowbird calls during nest-building to a group of nests in a defined spatial area (Forsman and Martin 2009).

Each of these cues tests whether a particular stimulus is associated with subsequent elevation of yolk T in host eggs. However:

(1) models of birds and playbacks of calls are not as powerful a stimulus as a live bird, and songbirds quickly habituate to both models and playbacks that are not accompanied by a live bird. Consequently, lack of elevated yolk T in host eggs after such a presentation may reflect the inadequacy of the experimental stimulus. Field observations of cowbirds perched near host nests and watching nest-building should be noted (Hahn et al. 1999), because such natural experiments can be used to evaluate the effect of such threats on host use of maternal effects.

(2) experimentally adding a cowbird egg to a host nest is a test whether that stimulus has an effect on yolk T of host eggs laid >24 hours later. Experimentally adding eggs should be tested in conjunction with presentation of models and/or playbacks of cowbirds, since arrival of a cowbird egg alone may not be sufficient stimulus if the host birds have not seen cowbirds near their nest. As noted above, models and playbacks are not as potent a stimulus as a live cowbird watching the nest, so lack of a host response cannot be definitively interpreted as the threat of parasitism having no effect.

Cowbird eggs in multiply parasitized nests: Our findings suggest that it would be fruitful to prioritize two approaches in future studies of maternal effects in multiply-parasitized nests:

(1) utilize genetic techniques to establish maternity of eggs in multiply-parasitized nests. This can facilitate more accurate comparisons of yolk T in singly- vs multiply-parasitized nests by guiding the use of “nest” or “egg” as the unit of analysis (*see Methods 2.4.2.1*). When used in conjunction with a banded, known age population, genetic analysis can also determine whether first-year breeders contribute a higher percentage of eggs to multiply-parasitized nests (*section 4.3* as McLaren et al. (2003) suggested), and whether their eggs are characterized by elevated yolk T.

(2) closely monitor nests to determine the lay order of cowbird eggs in multiply-parasitized nests. We did not know lay order of all multiply-parasitized nests, since 2 cowbird eggs can be laid by different females on the same day, and we checked nests only once daily. This would determine whether elevated yolk T is associated with only the 2nd (and 3rd) eggs. Good quality camera surveillance of nests will be necessary so that eggs can be distinguished based on color and speckle pattern.

Once genetic information and lay order information from natural experiments of parasitized nests is known, the design and interpretation of experiments involving multiply parasitized nests will be more productive.

Nest surveillance cameras We hypothesize that accelerated embryonic and nestling growth occur in cowbirds and their hosts as the result of elevated yolk T. Surveillance cameras at nests can provide precise information on length of incubation period and of nestling period of both host and cowbird young if eggs are allowed to hatch. This will allow comparison of rate of embryo development of (i) host eggs in nests that are parasitized vs unparasitized and (ii) cowbird eggs in singly vs multiply-parasitized nests, and these comparisons that will reveal if differences are consistent with the effects of elevated yolk T that have been observed in other species.

5 Conclusions

Maternal androgens have not previously been identified as factors in the arms race between avian brood parasites and their hosts. We found evidence that small songbird species use elevated yolk testosterone as a host defense against cowbird parasitism. This is also the first report that avian host species use a *tolerance defense*¹ (Raberg et al. 2007) against brood parasitism.

¹ Tolerance defenses are those that limit the severity of a parasite’s impact without reducing the intensity of parasitism while resistance defenses reduce the intensity of parasitism.

Traditionally, only resistance defenses -- such as ejecting or burying the cowbird egg or abandoning the nest to cowbird parasitism -- have been reported. We suggest that exploration of the use of tolerance defenses against cowbird parasitism is a promising area of future research in small host species that experience greatest reduction in success due to cowbirds.

We also found evidence that maternal effects are used by brood parasites as a defense against intra-specific competition for critical breeding resources, analogous to their use by non-parasitic avian species. We suggest that a promising area of future research is the examination of yolk T levels of cowbird eggs in both singly- and multiply-parasitized nests in combination with genetic analysis of the relatedness of all cowbird eggs at the study site.

There is conservation value in fully understanding the defenses used against cowbirds by forest-nesting neotropical migrants like our study species, since they are under pressure on both wintering grounds and breeding grounds due to loss of habitat (Terborgh 1989).

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APPENDIX

The basic Goodness of Fit (GoF) in maximum likelihood analysis is like the R^2 statistic from the least squares analysis in some respects. The only value that has meaning is how GoF statistics change as you fit more complex models.

For Goodness of Fit (GoF) analysis, we considered two models: the first model assumed that eggs were independent and used “egg” as the experimental unit; the second model assumed that eggs within nests were correlated and used “nest” as the experimental unit. We used the Bayesian Information Criterion (BIC) (Mayfield 1992, Jette, Hayden et al. 1998, DeCapita 2000, Hayden, Tazik et al. 2000, Kus and Whitfield 2005, Summers, Kostecke et al. 2006). There is no consensus on the magnitude of change in the GoF statistic that indicates whether the default or alternate model should be used. In the literature, recommendations range from 0 to 2 - 4 units as the threshold indicating which model should be used.

We ran the GoF analysis for the eggs of the three host species. For American redstart, the GoF analysis found no difference in BIC between the two models, and the correlation of eggs within-nests was very small (Appendix, Table 4). Consequently, the use of the simpler, independent model, in which “egg” is unit of analysis, is supported. We carried out the comparison of redstart eggs twice, using “egg” as the experimental unit as well as “nest”. The results from both analyses are presented in Table 2.

For veery, when the GoF was carried out, the BIC strongly indicated that the correlated model using “nest” as the experiment model should be used (Table 2). Consequently, we ran the analysis only once, using “nest” as the experimental unit. The results are presented in Table 2.

For red-eyed vireo, the GoF analysis yielded inconclusive results, because the GoF statistic is less than 2 and falls in the zone where there is no consensus regarding which model should be used. The authors believe that the use of “egg” as unit of analysis can be supported, because (i) the independent model using “egg” as unit of analysis is the simpler model; (ii) the decrease in BIC is a small value, less than 2; and (iii) the correlation of eggs within-nests is not large (Appendix, Table 4). Thus, as with the redstart, we carried out the comparison twice, once using “nest” as the unit of analysis, once using “egg” as the experimental unit. The results of both analyses are presented in Table 2.

Appendix, Table 1. Goodness of Fit tests

Host species	Correlation	Independent Model GoF	Correlated Model GoF	Difference	Choice of Model
American redstart	0.19	5.1	5.1	0.0	No difference in fit
Red-eyed vireo	0.31	5.7	3.8	1.9	Correlated model marginally better
Veery	0.87	21.0	-17.9	38.9	Correlated model much better

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Appendix: Table 1. Goodness of fit tests

Table 1. General comparison of size differences and parasitism rates among study species ^a

Species	American Redstart	Red-eyed Vireo	Veery	Brown-headed Cowbird
Adult size (g) Male Female	8.8 ± 0.5 8.7 ± 0.6	20.7 ± 2.8 19 ± 3.2	32.4 32.4	47.5 37.6
Egg size (mm)	16.2 x 12.3	20.4 x 14.8	22.9 x 16.9	21.5 x 16.4
Incubation period (days)	11-12	12.8	12	11-12
Nestling period (days)	9	10-12	10-12	9-10
Parasitism rate at study site	27.2%	58.8%	45.5%	NA

^a Size data taken from Cimprich (2000), Schwarz (1978), Ehrlich et al. (2000), Moskoff (1988), Sherry and Holmes (2005), Lowther (1997). Parasitism rates are data from our study site in the present study.

Table 2. Host eggs: comparison of yolk testosterone concentration in parasitized vs. unparasitized nests

Species	Unparasitized nests	Parasitized nests	Mann-Whitney- Wilcoxon	P-value
1. American redstart				
A. Eggs (N)	26	11		
Median testosterone (eggs)(ng / g)	13.1	16.8		
			267.5	0.014
B. Nests (N)	8	3		
Median testosterone (nests)(ng / g)	13.3	17.8		
			27	0.041
2. Red-eyed vireo				
A. Eggs (N)	14	20		
Median testosterone (eggs) (ng / g)	4.8	7.3		

			185.0	0.009
B. Nests (N)	4	6		
Median testosterone (nests)(ng / g)	5.5	8.8		
			15	0.083
3. Veery ^a				
A. Nests (N)	6	5		
Median testosterone (nests) (ng / g)	5.5	2.7		
			26.0	0.739

^a Veery eggs were compared using only one analysis, “nest”, because goodness-of-fit statistics did not support the using “egg” for veery eggs (see Appendix, Table 1).

Table 3. Cowbird eggs: comparison of yolk testosterone concentration in singly-parasitized vs. multiply-parasitized nests

	Cowbird eggs	Parasitized nests			Mann- Whitney- Wilcoxon	P-value
			Singly- parasitized Nests	Multiply- parasitized Nests		
1. Cowbird egg						
No. Eggs	24		13	11		
Median testosterone (eggs) (ng / g)			3.3	6.4		
					172.0	0.024
2. Parasitized nests						
No. Nests		17	13	4		
Median testosterone (nests) (ng / g)			3.3	6.0		
					50	0.063

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Cowbird-host hormone

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Table 1. General comparison of size differences and parasitism rates among study species ^a

Species	American Redstart	Red-eyed Vireo	Veery	Brown-headed Cowbird
Adult size (g) Male Female	8.8 $\pm$ 0.5 8.7 $\pm$ 0.6	20.7 $\pm$ 2.8 19 $\pm$ 3.2	32.4 32.4	47.5 37.6
Egg size (mm)	16.2 x 12.3	20.4 x 14.8	22.9 x 16.9	21.5 x 16.4
Incubation period (days)	11-12	12.8	12	11-12
Nestling period (days)	9	10-12	10-12	9-10
Parasitism rate at study site	27.2%	58.8%	45.5%	NA

^a Size data taken from Cimprich (2000), Ehrlich et al. (1988), Moskoff (2005), Sherry and Holmes (1997), Lowther (1993). Parasitism rates are data from our study site in the present study.

Table 2. Eggs of host species in parasitized and non-parasitized nests

Species	Non-parasitized nests	Parasitized nests	Mann-Whitney- Wilcoxon	P-value
1. American redstart				
A. Eggs (N)	26	11		
Median testosterone (eggs)(ng / g)	13.1	16.8		
			267.5	0.014
B. Nests (N)	8	3		
Median testosterone (nests)(ng / g)	13.3	17.8		
			27	0.041
2. Red-eyed vireo				
A. Eggs (N)	14	20		
Median testosterone (eggs) (ng / g)	4.8	7.3		

			185.0	0.009
B. Nests (N)	4	6		
Median testosterone (nests)(ng / g)	5.5	8.8		
			15	0.083
3. Veery ^a				
A. Nests (N)	6	5		
Median testosterone (nests) (ng / g)	5.5	2.7		
			26.0	0.739

^a Veery eggs were compared using only one analysis, “nest”, because goodness-of-fit statistics did not support the using “egg” for veery eggs (see Appendix, Table 1).

Table 3. Cowbird eggs in singly-parasitized vs multiply-parasitized nests

	Cowbird eggs	Parasitized nests			Mann- Whitney- Wilcoxon	P-value
			Singly- parasitized Nests	Multiply- parasitized nests		
1. Cowbird eggs						
No. eggs	24		13	11		
Median testosterone (eggs) (ng / g)			3.3	6.4		
					172.0	0.024
2. Parasitized nests						
No. nests		17	13	4		
Median testosterone (nests) (ng / g)			3.3	6.0		
					50	0.063