

The ability to mount multiple immune responses simultaneously varies across the range of the tree swallow

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Variation in immune responses is an important part of life history variation. When correlations between multiple immune measures are reported, studies report different patterns. I tested whether the correlation between levels of immune response was consistent across a species range. The ability of tree swallows *Tachycineta bicolor* to simultaneously produce immune responses to both a humoral immune response and T-cell mediated local inflammation to PHA was tested at three sites that span the breeding range. Females in Tennessee maintained stronger PHA responses than did females in either New York or Alaska. In New York and Alaska, individuals that produced strong PHA responses produced low levels of antibodies to a humoral challenge of sheep red blood cells (SRBC). However, in Tennessee, individuals that showed strong local PHA inflammation also mounted strong responses to SRBC. Increasing daily daytime temperatures led to increased PHA response, but there were no differences in the effect of temperature among sites. These results indicate spatial and/or temporal variation occurs in the ability to produce multiple immune responses simultaneously; this pattern suggests important geographic (or temporal) differences in factors driving investment in immune activity. In addition, these results suggest that studies extrapolating results across populations should be careful to consider geographic variation in immune activity.

Variation in immune defense is a component of life history variation, due in part to the selective pressures brought by parasites and pathogens on their hosts (Ricklefs and Wikelski 2002, Lindström et al. 2004, Ardia 2005b, Martin et al. 2006b). Assessing immune responses can be difficult because the vertebrate immune system is an inter-related system (Roitt et al. 1998) and encountering multiple threats may influence the ability of individuals to produce strong immune response simultaneously (Mosmann et al. 1986a, Graham 2002). Many studies report a negative correlation between antibody mediated immunity and cutaneous swelling in response to a mitogen, a generalized measure of cell-mediated immunity (Klein and Nelson 1999, González et al. 1999, Buchanan et al. 2003, Faivre et al. 2003). For example, house sparrows *Passer domesticus* producing strong humoral immune responses showed weak cell-mediated inflammation in response to a mitogen (Buchanan et al. 2003), while prior wounding reduced cell-mediated immune

responses in white-footed mice *Peromyscus leucopus* (Martin et al. 2006c). In poultry, mounting multiple immune responses limits the ability to respond effectively to each simultaneously (Dunnington et al. 1992, 1993, Eide et al. 1992), although in some strains of poultry artificial selection for increased antibody responses leads to increased cell-mediated immune activity (Parmentier et al. 1993). However, other studies have reported limited or no individual correlation depending on the immune measures compared (Martin et al. 2006b, Matson et al. 2006). Martin et al. (2006a, b) found no correlation between a humoral response to keyhole limpet hemocyanin (KLH) and delayed-type hypersensitivity response in house sparrows. Finally, some studies report positive correlations, such as between PHA response and humoral immune response in the bird family Hirundinidae (Møller et al. 2001). It remains unclear whether patterns of correlation between immune responses are consistent within a species or vary across space or over time.

Given the range of responses reported in other studies, I take advantage of a larger experiment on geographic variation in life history tradeoffs (Ardia 2005a, 2006) to test for geographic variation in the correlation between two immune challenges given simultaneously. I assessed individual variation in the ability of tree swallows *Tachycineta bicolor* to simultaneously produce multiple immune responses to an immune challenge to PHA and an inducible humoral immune challenge at three sites that span the breeding range. When multiple threats explicitly counteract such as occur with multiple infections (Graham et al. 2005) or between the two arms of the vertebrate acquired immune system (Martin et al. 2006c), one would predict a negative relationship between immune responses. However, cutaneous inflammation in response to PHA may better indicate condition (Hórák et al. 1999, Ewenson et al. 2001, Lifjeld et al. 2002) in addition to levels of T-cell proliferation (Martin et al. 2006a), thus positive correlations might be expected in high quality individuals or high quality locations. Given the possibility of counteracting effects of immune system stimulation, I tested 1) whether individuals mounting strong cutaneous inflammation would also mount strong humoral responses and 2) whether this relationship varied across the breeding range.

Individuals may differ among sites in their investment in immune function because environmental conditions influence both exposure to health threats (e.g. selection for immune investment and suite of immune stressors) and resource availability (e.g. condition). A recent common garden experiment revealed strong differences in cell-mediated immune function between geographically widespread populations of house sparrows *Passer domesticus* (Martin et al. 2004); these results were supported in a study comparing other immune measures in the same populations (Martin et al. 2006b). I test whether immune responses in tree swallows show similar variation within a species by comparing immune responses at three sites from 35°N to 64°N. As individuals inhabiting warmer areas are believed to have higher disease exposure (Hamilton and Zuk 1982, Møller 1998, Møller et al. 1999), I predicted that the immune responses of females breeding in the southern portion of the range would be positively correlated, perhaps due to greater selection for increased immune function and the indication that southerly individuals are in higher condition (Ardia 2005b). Because the work was conducted at each site in different years, it is possible that differences in immune responses may reflect yearly, rather than geographic variation. Large differences in environmental driving forces among sites lead to limited intra-year correlation in conditions (Ardia 2006), suggesting that measuring sites in different years does not greatly minimize comparisons. However, without multiple years at each

site, it is difficult to explicitly exclude yearly variation as the cause of the pattern reported here. Regardless, differences among sites or years would indicate that variation in the correlation among immune responses occur, whether temporal or spatial, as well as the environmental variables that drive these differences.

I assessed immune response through two common methods in wild vertebrates: 1) innate induced immune function, measured as cutaneous cell-mediated immune activity to a mitogen (Martin et al. 2006a) and 2) humoral immunity, measured as antibody production to sheep red blood cells (SRBC), as mammalian red blood cells are recognized by birds as a novel antigen. Cutaneous cell-mediated immune activity is often characterized by a non-specific inflammatory response as heterophils and macrophages release cytokines that stimulate lymphocytes and macrophages to infiltrate a site, causing localized swelling (Stadecker et al. 1977, Goto et al. 1978). A frequent test of cutaneous cell-mediated immunity is via exposure to a non-specific mitogen, in this case phytohaemagglutinin (PHA), which causes a localized immune response (Parmentier et al. 1998, Smits and Williams 1999). By contrast, humoral immunity assesses B-cell differentiation and specialization to produce specific antibodies to a recognized antigen (Roitt et al. 1998, Davison 2003).

Methods

General field methods

Tree swallows *Tachycineta bicolor* are a widespread and common cavity-nesting North American migrant member of the family Hirundinidae (Robertson et al. 1992). Tree swallows breeding in nestboxes erected for their use were studied from May to July 2000 in Tompkins County, New York, USA (centered on 42°29'N, 76°27'W), from April to July in 2001 in Loudon, Anderson, and Knox Counties, Tennessee, USA (centered on 35°53'N, 84°18'W) and May to July in 2002 near Fairbanks, Alaska, USA (centered on 64°49'N, 147°52'W). The females monitored here were part of a larger study examining tradeoffs between reproductive effort and offspring quality through a brood size manipulation (Ardia 2005a, b); however, only control females who raised broods unchanged in brood size are considered here (Alaska n = 16, New York n = 17, Tennessee n = 17).

Nests were checked daily during egg laying to determine date of clutch initiation and clutch size. Nest visitation rates were recorded to include provisioning behaviour as a covariate in analyses; three 60-min behavioural observations were conducted using video cameras between days 8–11 of the nestling period for

each breeding pair; observations were balanced amongst nests and sites across the sampling period. Sexes were differentiated in video observations by marking females with a small dot of white paint on the back. Insect availability was recorded using a 2-m aerial insect sampler powered by a Robbins and Myers 1650 r.p.m. (12.95 m s^{-2}) 1/12 HP motor (Dayton, OH, USA) (McCarty and Winkler 1999) to collect daily samples of aerial insect abundance during the breeding season.

Breeding females were exposed to PHA and SRBC on day 4 and then recaptured on day 6 to determine PHA response and day 12 to determine antibody production to SRBC. Repeated capture had no effect on female body mass, abandonment rate or nestling quality (Ardia 2005a, b). Female tree swallows can be aged reliably by plumage as second year or after-second-year; this allows for age to be included as a covariate due to differences in reproduction between age groups (Winkler and Allen 1996). Blood was drawn from the brachial vein (50 to 150 μl) to determine pre-exposure levels of antibodies to sheep red blood cells (from 20 μl plasma) and each bird was injected intraperitoneally with 5×10^7 sheep red blood cells (ICN Biomedicals, Aurora, OH) suspended in 100 μl PBS (Deerenberg et al. 1997). All individuals were captured 8 d later, the time of peak antibody production in tree swallows (Ardia unpubl.), to draw blood to determine post-exposure primary antibody titers in plasma. Antibody titers were measured in all blood samples using a base-2 serial dilution haemagglutination test conducted with 20 μl of plasma on 96-well microtitre plates (Roitt et al. 1998). Samples were serially diluted starting with 20 μl PBS and to each well 20 μl of a 2% suspension of SRBC in PBS was added. Plates were incubated at 37° for 1 h. Titers are given as the \log_2 of the reciprocal of the highest dilution of plasma showing positive haemagglutination. No individuals showed pre-exposure antibodies, so post-exposure antibody levels are used in analyses. For each plate, positive and negative controls were included.

On nestling day 4, 0.15 mg of phytohaemagglutinin (PHA-P) in 30 μl PBS was injected into the left patagial wing web of breeding females (Smits et al. 1999). Prior to each injection, the thickness of the wing web was measured three times using a digital micrometer and reported as the average thickness. Individuals were recaptured and remeasured ca 48 h after injection and wing web thickness was remeasured three times and the average measurement used (repeatability: pre-exposure $F_{49,50} = 5.34$, $p = 0.001$, $R = 0.85$, $R^2 = 0.94$, post-exposure $F_{49,50} = 3.76$, $p = 0.01$, $R = 0.81$, $R^2 = 0.86$) (Lessells and Boag 1987). While many studies assess PHA response after 24 h (Smits et al. 1999), pilot data suggested that responses after 24 and 48 h differed similarly from controls and there was no difference in

swelling response between 24 and 48 h ($t_{1,42} = 0.67$, $p = 0.50$) and thus PHA response after 48 h was measured in order to minimize disturbance to breeding birds. Cell-mediated immune response was considered to be the ratio of post-injection thickness to pre-injection thickness (Smits et al. 1999); results are similar when linear swelling measures are used. Initial wing web thickness was included as a covariate to control for individual variation. Research has found that control injections of PBS are not necessary (Smits et al. 1999). All work was conducted with approval of the Institutional Animal Care and Use Committee of Cornell Univ. (Protocol #99-37-02).

Analysis

Variation in immune responses among breeding sites were examined using a General linear model (PROC GLM) (Anon. 1988). In addition to the central hypothesis of comparing breeding locations, measures of individual variation/condition in tree swallows were included as covariates: clutch initiation date, female age, and flattened straightened third primary wing length (Winkler and Allen 1996) and feeding visits h^{-1} (see Ardia 2006 for more information), brood size, residual body mass (body condition), and initial wing web thickness as immune responses can be condition-dependent (Lochmiller and Deerenberg 2000, Norris and Evans 2000). Three environmental variables, intended to control for environmental variation, were calculated for each individual over the 48 h period of PHA measurements: mean insect availability, mean daily daytime temperature, and mean nighttime temperature. Body condition was calculated as the residual of a regression of tarsus length vs body mass. Because of differences in timing of breeding among sites, clutch initiation date was standardized to a mean of 0 for each site; results are similar when analyses are conducted with day of year in models. Only females making their first breeding attempt of the season were used. Year was not included in models, because in addition to being redundant with breeding site, the geographically-separated sites used in this experiment show no correlation in environmental conditions during a calendar year (Ardia 2006).

First, factors predicting immune response to PHA were assessed with the following variables in the general linear model: standardized clutch initiation date, female age (second-year individuals vs after-second-year individuals), brood size, feeding visitation rate, wing feather length, residual body mass, initial wing web thickness, insect availability, temperature, and breeding site. Second, factors predicting antibody production to SRBC were analyzed with the covariates listed above, as well as the PHA response of each female. SRBC

responses were modeled with PHA as a covariate rather than the reverse because the resting T-cell population stimulated by exposure to PHA is also responsible in part for mediating antibody responses and because PHA responses in this design occur first in time relative to the period of peak antibody production, making PHA response a more relevant predictor of SRBC antibody production than the converse (Roitt et al. 1998); results were similar when SRBC response is included as a covariate in models predicting PHA responses. All two-way interactions with $p > 0.20$ were removed sequentially in order of highest p-value; removing nonsignificant interactions did not affect overall significance of main effects. Significant differences among sites were compared using least square means.

Results

Immune responses to PHA of breeding female tree swallows followed a latitudinal gradient, with females in the southern portion of the range (Tennessee) producing stronger responses than did birds in the center (New York), which in turn maintained stronger PHA responses than did females breeding in the northern portion of the range in Alaska (all F values reported are from full models including all covariates described in Methods; site effect $F_{2,36} = 8.18$, $p = 0.001$, Fig. 1a). Females breeding later in the breeding season produced weaker immune activity to PHA than did females breeding earlier in the breeding season across all three sites ($F_{1,36} = 6.65$, $p = 0.015$; standardized parameter estimate -0.056). Brood size, wing length, residual body mass, feeding effort, food availability, and female age had no effect on immune activity following exposure to PHA ($F_{1,36} \leq 0.50$, $p \geq 0.40$). Daily daytime temperature had a marginal effect on PHA responses; birds mounting PHA responses during

warmer weather tended to produce stronger PHA responses ($F_{1,36} = 3.13$, $p = 0.08$). There was no interaction between the effect of temperature and PHA response among sites ($p > 0.20$).

In contrast to the latitudinal variation in PHA, there was no difference between sites in antibody production to SRBC ($F_{2,32} = 1.67$, $p = 0.20$, Fig. 1b). As with the response to PHA, clutch initiation date predicted antibody production, with early-laying individuals producing more antibodies than do later-laying birds ($F_{1,32} = 6.01$, $p = 0.02$, standardized parameter estimate -0.35). Clutch size, wing length, residual body mass, feeding effort, temperature, food availability, and female age had no effect on immune activity following exposure to SRBC ($F_{1,35} \leq 1.52$, $p \geq 0.3$).

The ability of individuals to produce antibodies following exposure to SRBC was predicted by swelling response to PHA ($F_{1,32} = 5.88$, $p = 0.02$), but the direction of this effect varied by site (interaction between PHA response and breeding site $F_{2,32} = 8.3$, $p = 0.007$). In Alaska and New York, individuals producing strong responses to PHA mounted lower responses to SRBC (Fig. 2; Alaska $\beta = -0.22$, $R^2 = 0.21$, $p < 0.01$, New York $\beta = -0.11$, $R^2 = 0.23$, $p < 0.001$), while in Tennessee, breeding females who mounted strong PHA responses also produced high levels of antibodies to SRBC ($\beta = 0.16$, $R^2 = 0.27$, $p < 0.001$).

Overall, differences among sites were not due to differences in provisioning behaviour, as breeding females made similar numbers of feeding visits regardless of breeding site (mean visits $\text{min}^{-1} \pm \text{SE}$: Alaska 0.14 ± 0.15 , $n = 16$; New York 0.18 ± 0.07 , $n = 17$; Tennessee 0.15 ± 0.11 , $n = 17$; $F_{2,44} = 0.36$, $p = 0.55$). In addition, there was no difference among sites in clutch size (mean clutch size $\pm \text{SE}$: Alaska 5.61 ± 0.13 , $n = 16$; New York 5.63 ± 0.16 , $n = 17$; Tennessee 5.51 ± 0.17 , $n = 17$; $F_{2,33} = 0.27$, $p = 0.60$) or in

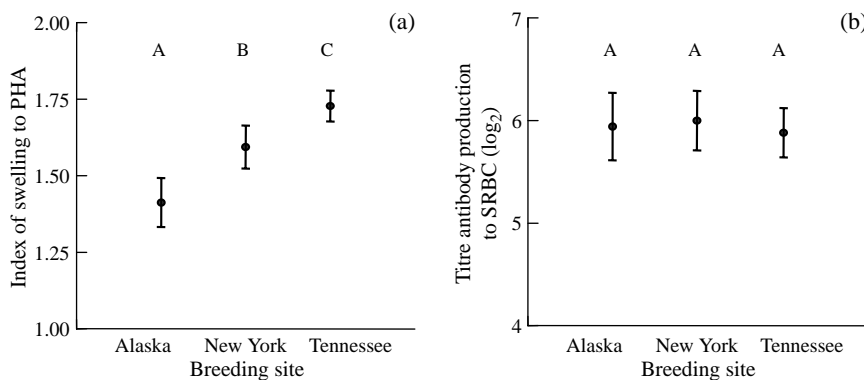


Fig. 1. Immune response to (a) phytohaemagglutinin (PHA) and (b) sheep red blood cells (SRBC) of female tree swallows across their range. Means are least square means and error bars represent standard error. Letters above means represent significant differences at $p < 0.01$. Sample sizes: Alaska = 16, New York = 17, Tennessee = 17.

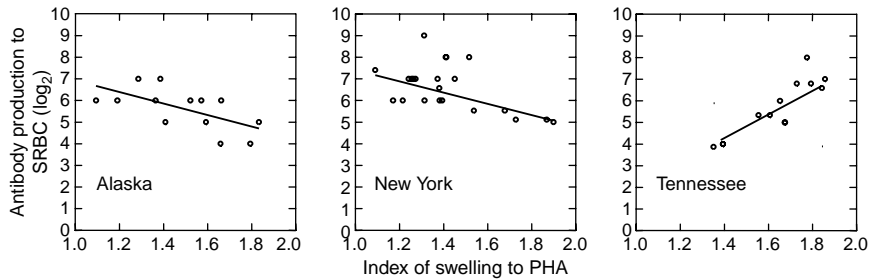


Fig. 2. Immune responses of breeding female tree swallows to a simultaneous immunochallenge to the mitogen phytohaemagglutinin (PHA) and the novel antigen sheep red blood cells (SRBC) at multiple sites across their breeding range.

number of nestlings raised (mean nestlings on day 10 \pm SE: Alaska 5.19 ± 0.18 , $n = 16$, New York 5.24 ± 0.13 , $n = 17$, Tennessee 5.18 ± 0.17 , $n = 17$).

Discussion

Here I report variation in the relationship between multiple immune responses within a species similar to that reported among species. Both positive and negative correlations were found when examining sites across the range of a widespread species, the tree swallow. In two of three sites, breeding female tree swallows producing strong cutaneous swelling in response to PHA mounted weak humoral responses to SRBC. However, the relationship varied, as the third site showed a positive correlation between cutaneous swelling and antibody production. This result suggests that the relationship between multiple immune challenges can vary over space or at least over time. Differences among sites in immune response may occur for a variety of reasons. For example, females at different sites could have differences in immune system architecture, such as sensitivity in leucocytes, ability to recognize SRBC or different activity level in cytokines. This proximate variation may be underlain by nonadaptive genetic differences or may occur because of adaptive pressures on the immune system. Ecological immunology theory posits that the immune system tradeoffs with other life history traits, such as reproduction and growth (Ricklefs and Wikelski 2002). In a parallel study, I found that return rates and patterns of parental investment differed among sites, suggesting Tennessee tree swallows have higher survival rates (Ardia 2005b). Individuals surviving longer would be expected to invest more in traits that enhance survival (Hamilton 1966, Tella et al. 2002), such as immune function. Because I did not explicitly measure disease exposure, it is difficult to differentiate between the effects of disease exposure or allocation differences. This is a nontrivial matter, as higher immune function could arise because individuals are selected to invest heavily in immune function due to

higher threats from parasites or because individuals with higher survival invest more in self-maintenance or both.

The breeding sites studied differ in factors that could directly influence two critical factors driving immune response: condition and disease exposure. Both temperature and food supply can influence body condition and thus allocation to immune response, particularly PHA response. Females at all three sites showed a trend towards increased PHA responses with increasing daily temperatures, supporting the idea that PHA responses are in part condition-dependent. However, daily temperatures alone cannot explain the patterns reported here. Temperatures during the period of immune activity stimulated in this study did not differ among sites (New York 16.8°C , Alaska 16.2°C , Tennessee 17.2°C). Food resources are considerably higher in Alaska than in Tennessee or New York (Ardia 2006); regardless there was no predictive effect of food supply during the period of immune activity on immune responses.

An important potential contributor to site level difference could be the length of the breeding season. In Alaska, the time between arrival and dispersal of adults is ca 8 weeks, while in New York the period may last over 16 weeks and in Tennessee 24–26 weeks (Ardia unpubl.). Thus the time period that adults are exposed to disease in the breeding grounds varies widely. Parasite levels can vary geographically (Sol et al. 2000, Freeman-Gallant et al. 2001, Gibb et al. 2005) and there is some suggestion that parasite prevalence appears to be higher in warmer areas (Møller 1998, Møller et al. 1999). However, without a detailed survey, the role of parasite pressure remains speculative. Tree swallows winter across a wide area of the southern North America and Central America, little is known about affinity for particular wintering areas (Robertson et al. 1992). Because each site has only a single year of data, the differences reported here may represent yearly environmental variation rather than true site-level differences, in which cases yearly variation in parasite levels could drive these patterns.

Another important difference among sites may be in the influence of hormones on immune response. Hormones, particular stress hormones such as

corticosterone, can be immunosuppressive (Gross and Siegel 1983, Wilckens and de Rijk 1997, Svensson et al. 1998). Within species, stress hormone levels can vary geographically (Silverin et al. 1997), as can their effect on immune activity (Martin et al. 2005). Thus variation in immune responses can be caused simply through differential action of stress hormones; however, this may only be a proximate mechanism that underlies adaptive variation in immune response. In addition, the handling regime in this study involved capturing birds three times, thus contributing to high corticosterone levels and site-level differences if sites differed in the sensitivity to handling.

Negative correlations may occur because the immune activity levels measured represent inherent population levels and negative correlations represent negative genetic co-variance (Lazzaro et al. 2004). If this is the case, the levels of immune response reported here would be similar whether multiple immune challenges were faced or not. An alternative explanation is that producing multiple immune responses disrupts a balance within the immune system, such as might occur between the two arms of the vertebrate acquired immune system: cell-mediated vs humoral immunity. When one type of T-helper cell is stimulated it releases cytokines, intercellular signals, that serve to both stimulate an immune response to the recognized threat and to downregulate the other arm of the acquired immune system (Mosmann et al. 1986b, Graham 2002). The generalized cutaneous swelling response to PHA assessed here is not a true measure of cell-mediated immunity, thus it is unlikely that the pattern reported is due simply to cross-regulation of the immune system. The positive correlation reported in Tennessee suggests that immune system synergy might also play a role. Similar differences within a species have been reported between breeds of cattle, where Angus steers showed both stronger humoral and cell-mediated immune responses relative to Simmental steers, possibly due to differences in breeding regime and longevity (Engle et al. 1999). However, for the same reason mentioned above, comparison of multiple to single responses is needed to address whether synergistic activation plays a role. In addition, if the patterns reported here represent yearly environmental variation within a site, then negative correlations may simply reflect condition-dependent variation and not population differences in genetics. Further data are needed to properly address this possibility.

The immune system challenges used in this study assess markedly different components of the vertebrate immune system. Cutaneous swelling in response to PHA assesses induced innate immune activity. The reaction to PHA involves multiple cellular processes, is dynamic, and the populations of cells recruited to the sites varies over time; without tissue samples it is

difficult to precisely assess the actual T-cell infiltration of the exposure site (Stadecker et al. 1977, Martin et al. 2006a). For example in house sparrows, basophil infiltration peaks 48 h following exposure, while heterophils peak at 12 h and decline to almost baseline at 48 h (Martin et al. 2006a). A greater level of swelling may indicate different populations of cells in each population sampled. This makes conclusions that populations (or individuals) with similar or different levels of swelling have similar or different levels of T-cell mediated immune activity problematic. Humoral immune response to SRBC, by contrast, is a measure of inducible immunity and reflects antibody production following recognition of SRBC as a novel antigen. Because humoral immunity rarely involve systemic inflammation it is believed to be less costly (Klasing and Korver 1997), though more research is needed before the greater energetic cost of PHA responses can be conclusively determined (Martin et al. 2003).

In summary, the results reported here for New York and Alaska demonstrate a limited ability in individuals to mount multiple immune responses simultaneously, though they may not necessarily represent a limited ability to simultaneously activate the immune system. These differences are likely underlain by differences in condition among sites, as well as differences in the selective regimes driving immune system investment or at minimum represent yearly variation in immune system correlations. The next step is to compare multiple responses to single responses in the same population to determine whether tradeoffs exist and whether these results are consistent over time within a site. Lastly, these results suggest that studies extrapolating results across populations should be careful to consider geographic or yearly variation in immune activity.

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