A shared chemical basis of avian host–parasite egg colour mimicry

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Avian brood parasites lay their eggs in other birds' nests and impose considerable fitness costs on their hosts. Historically and scientifically, the best studied example of circumventing host defences is the mimicry of host eggshell colour by the common cuckoo (*Cuculus canorus*). Yet the chemical basis of eggshell colour similarity, which impacts hosts' tolerance towards parasitic eggs, remains unknown. We tested the alternative scenarios that (i) cuckoos replicate host egg pigment chemistry, or (ii) cuckoos use alternative mechanisms to produce a similar perceptual effect to mimic host egg appearance. In parallel with patterns of similarity in avian-perceived colour mimicry, the concentrations of the two key eggshell pigments, biliverdin and protoporphyrin, were most similar between the cuckoo host-races and their respective hosts. Thus, the chemical basis of avian host–parasite egg colour mimicry is evolutionarily conserved, but also intraspecifically flexible. These analyses of pigment composition reveal a novel proximate dimension of coevolutionary interactions between avian brood parasites and hosts, and imply that alternative phenotypes may arise by the modifications of already existing biochemical and physiological mechanisms and pathways.

Keywords: eggshell coloration; host–parasite similarity; mass spectrometry

1. INTRODUCTION

Avian brood parasites lay their eggs into nests of other species, and reduce or completely eliminate the breeding success of foster parents [1]. Such social parasitism emancipates parasites from the costs of parental care and imposes severe fitness losses on the hosts, resulting in an arms race [2] between hosts discriminating and rejecting parasitism, and parasites tricking or enforcing hosts to accept parasitic eggs and/or young [3–5]. For example, the common cuckoo (*Cuculus canorus*; hereafter: cuckoo) is an obligate brood parasite whose eggs have been found in greater than 125 songbird species' nests [6,7], although only few of them (approx. 15) are primary hosts and many of them are not suitable hosts at all [1,7]. The cuckoo has evolved several strategies to decrease or circumvent the rejection of its eggs by the hosts, including the laying of relatively small eggs [8], the formation of both absolutely and relatively thicker eggshells [9–11], and, perhaps its best-known adaptation, the avian-perceived mimicry of host egg colours and maculation patterns [12–14] (figure 1).

The many host species of the cuckoo lay eggs that vary vastly in appearance, and the accurate mimicry of different host egg morphologies is possible because the cuckoo has evolved a variety of distinct host-specific races (host-races: *gentes*) [15,16], with at least 15 distinct cuckoo egg phenotypes already described [7]. Each female cuckoo lays a consistent egg type [17], typically matching the egg colour and maculation of a locally predominant host species [18–20]. The rejection of foreign eggs by hosts, including parasitic cuckoo eggs, is negatively associated with the extent of both eggshell background colour similarity [21] and maculation matching [22], as demonstrated by analyses of similarity using human perception [23,24], full spectrum physical reflectance [25–27] and avian sensory modelling [12–14,28].

There is extensive comparative evidence that parasite–host coevolutionary arms races have affected the diversity of coloration and maculation of bird eggs in both hosts and parasites [29,30]. Yet, to date, the structural bases, including the chemical substrate, of avian-perceived similarities between host and parasite eggshell appearance remain unknown. We set out to assess the alternative hypotheses of perceptual versus structural mechanisms,

Hungary
great reed warbler
Acrocephalus arundinaceus

Finland
common redstart
Phoenicurus phoenicurus

Czech Republic
reed warbler
*Acrocephalus scirpaceus*
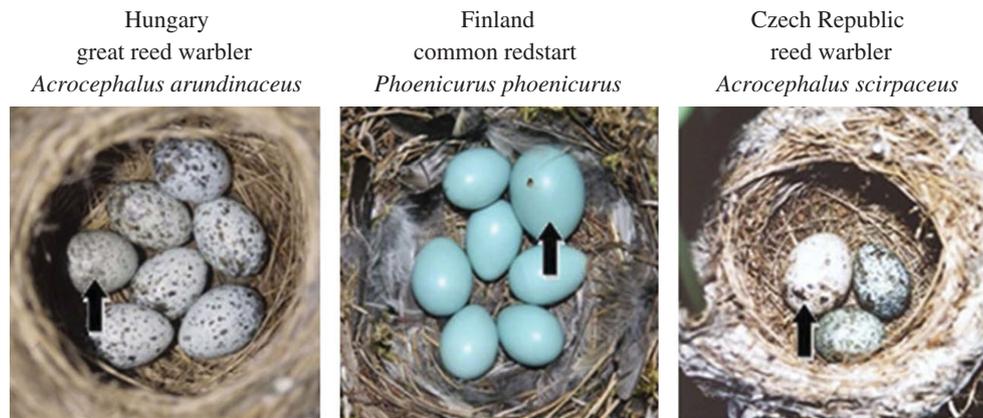
Figure 1. Representative nests with both host and parasitic common cuckoo eggs, illustrating mimicry of colour and maculation for the host–parasite systems included in our analyses. Black arrows identify the parasite egg. Photo credits: C. Moskát, T. Grim and M. Honza.

specifically whether the cuckoo generates mimicry based on different mechanisms than hosts, or replicates host eggshell appearance using the same set of pigments, and their concentrations, to achieve egg colour mimicry. Towards this aim, we used an integrative approach based on biochemical analyses of avian eggshell pigments from hosts, sympatric non-hosts and cuckoo host-races. The analysis of the chemical basis of egg colour mimicry in avian brood-parasites provides new avenues for investigating the general principles of host–parasite sensory coevolution, as it expands work into the proximate basis of the different sensory modalities of avian host–parasite mimicry, including visual [12,31], tactile [32,33] and acoustic [34,35] cues described in other host-mimetic lineages. For example, details of a potential chemical mechanism of avian eggshell mimicry also allow for extensive comparative analyses between the known chemically based mimetic and deceptive mechanisms of other host–parasite systems, including olfactory mimicry in socially parasitic insects [36–38], and the evolutionary alternatives of sensory deception in general [39].

Although bird eggs are some of the most diversely coloured natural materials [29,30], avian eggshell colours appear to be predominantly generated by two main pyrrol pigments: biliverdin (responsible for blue–green coloration) and protoporphyrin IX (responsible for brown–red maculation patterns; reviewed in [40]). These two pigments are found across all major lineages of avian diversity [41], including recently extinct birds [42]. Here, we analysed the concentration of these two pigments in eggshells of three mimetic cuckoo host-races, their respective local songbird host species and several sympatric non-host songbirds. Based on the known limited diversity of avian eggshell pigments [40], we predicted that eggshell pigment concentrations of mimetic cuckoo host-races would be more similar to local hosts' eggs compared with either local non-host eggs, non-local host eggs or eggs of other cuckoo host-races.

2. MATERIAL AND METHODS

(a) *Sample collection*
All sample collection was approved by our local governmental and institutional research permits. We conducted fieldwork in 2006–2008 in three localities in Europe

(Hungary, Finland and Czech Republic) to source eggshells from three different cuckoo host-races and their different locally parasitized main host species (table 1). Whenever possible, eggs of hosts and parasites were collected from the same nest, and eggs of non-hosts (or the original primary local host, the great reed warbler *Acrocephalus arundinaceus*, in the case of Czech samples; see table 1) were collected in sympatry.

Fresh, unincubated eggs were opened and cleaned with distilled water and then with 70 per cent ethanol, and stored in a cool dark place until spectral measurement and pigment extraction in 2009. In our statistical analyses (see §2*e*), we ran pairwise post hoc comparisons of each of the host and non-host species, and of cuckoo host-races globally, to provide specific tests of our predictions regarding repeated evidence of perceptual and chemical similarities between host and host-specific cuckoo eggs.

(b) *Spectral measurements*
We first set out to confirm perceptual similarity in eggshell coloration between cuckoo host races and the sampled host species, relative to non-host species, using avian-perceived sensory modelling techniques [12,28]. We followed our published definitions and methodology [42] for reflectance measurements of eggshell background coloration across the wavelength range 300–700 nm at three random equatorial locations of eggshell avoiding maculation. Measurements were taken using an Ocean Optics USB2000 miniature fibre optic spectrometer, connected to a portable computer, illuminated by a DT mini-lamp and OOIBase32TM operating 136 software (Ocean Optics, Inc., Dunedin, FL, USA). All measurements were taken at a 90° angle to parallel the practice recommended in some of the technical literature [42], so as to provide data that are comparable with previous work on the physical and perceptual bases of cuckoo-host egg colour similarity and cues for rejection behaviours [22,25,28]. White and dark standard reflection calibration measurements were taken every three measurements using an Ocean Optics WS-1 diffuse reflectance standard and a miniature cardboard box, respectively.

(c) *Perceptual modelling*
Perceivable differences between cuckoo and host/non-host egg colours, as seen through an avian host's eye, were estimated using the sensory model developed for tetrachromatic vision [45]. Most, but not all, oscine Passeriforme taxa are
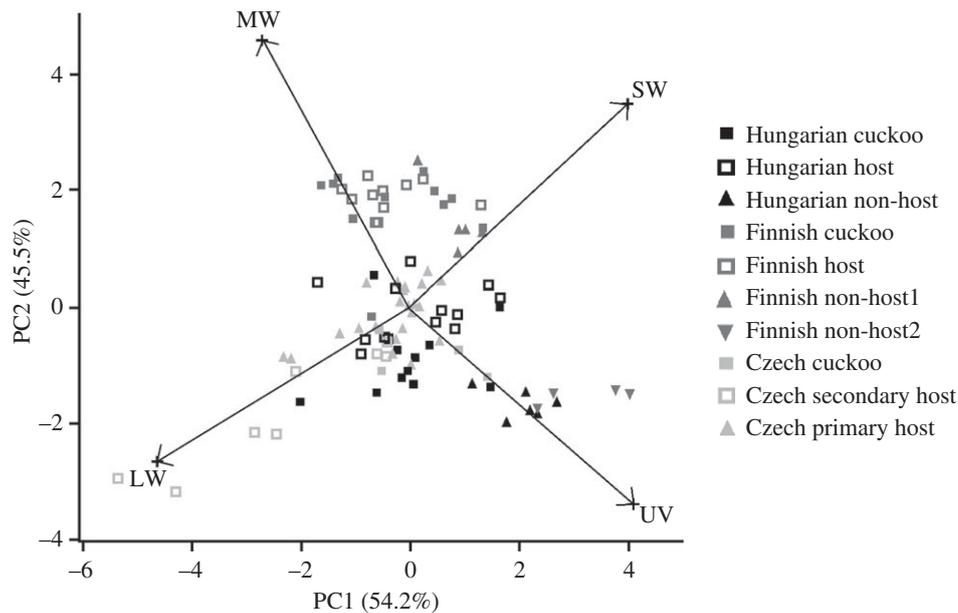
Figure 2. Principal component (PC) biplot showing projections of eggshell background coloration in avian colour space (points) and projection of eigenvectors (arrows) of proportionate excitation of the four avian chromatic photoreceptors. Eigenvalues for each PC are presented as percentage variance explained and illustrated on axis labels. LW, long wavelength; MW, medium wavelength; SW, short wavelength; UV, ultraviolet.

Table 1. Sample ID, species, nesting environment and number of samples used in each analysis.

ID	species	nest environment	colour analysis	pigment analysis
Hungarian cuckoo	common cuckoo, *C. canorus*		12	5
Hungarian host	great reed warbler, *A. arundinaceus*	open	14	5
Hungarian non-host	barn swallow, *Hirundo rustica*	closed	6	5
Finnish cuckoo	common cuckoo, *C. canorus*		12	5
Finnish host	common redstart, *P. phoenicurus*	closed	13	5
Finnish non-host1	pied flycatcher, *Ficedula hypoleuca*	closed	5	5
Finnish non-host2	great tit, *Parus major*	closed	5	5
Czech cuckoo[a]	common cuckoo, *C. canorus*		5	5
Czech secondary host[a]	reed warbler, *A. scirpaceus*	open	9	5
Czech primary host[a]	great reed warbler, *A. arundinaceus*	open	23	5

[a]In the Czech Republic, we sourced cuckoo eggs from nests of the reed warbler, but locally the cuckoo more often parasitizes great reed warblers [43,44].

considered to have ultraviolet-sensitivity (UVS) in one of their two types of short wavelength- (SW-) sensitive cones [46]. Owing to the lack of focal data on cuckoo host species' own cone sensitivities, the parameters that we used were those reported for a UVS passerine, the blackbird (*Turdus merula*) [47]. Model-specific parameters and procedures are described fully elsewhere [28,30,47]. The ability of a bird to discriminate between two colours is affected by the surrounding ambient light [45]. Therefore, irradiance enters as a critical parameter in the sensory models [12]. Open nest irradiance data were used for the colour space analyses (see below), whereas both open and closed nest irradiance spectra to simulate natural host and non-host nest light conditions (table 1) were used for the analyses of 'just noticeable difference' (JND) analyses. Open and closed nest irradiance spectra were extracted from [48] between 300 and 700 nm using VISTAMETRIX v. 1.35 software (available from http://www.skillcrest.com/). We used the Vorobyev–Osorio tetrachromatic perceptual model [45] using AVICOL v. 2 software [49] to calculate a bird's ability to distinguish between cuckoo and host egg coloration, measured as the quantum catch for each photoreceptor; four cones responsible for tetrachromatic

colour vision; and the combination of medium and long wavelength-sensitive cones (MW and LW, respectively), which have similar spectrum sensitivity properties of avian rod and cone photoreceptors [47], to assess achromatic or brightness perception. AVICOL also extracts JNDs for chromatic or achromatic vision.

We analysed differences in eggshell background coloration in avian tetrachromatic colour space by standardizing quantum catch of each cone by the total quantum catch of the four cones responsible for colour vision [28]. We then conducted principal component (PC) analysis on a correlation matrix of these colour space data to extract two orthogonal variables that explain a majority of the original variance (figure 2). We compared eggshell coloration and brightness between groups using general linear mixed models (GLMMs) with either of the first two colour space PC scores and the combined excitation of MW and LW cones (brightness) as responses, species/host-race ID as fixed effect and nest ID nested within year of egg collection as a random effect.

To analyse perceived eggshell colour differences while taking into account increases in photoreceptor error resulting from distinguishing similar colours [45,50], perceptual

distances of colours were also analysed as JNDs for both chromatic (ΔS) and achromatic (ΔfQ) components of colour vision. A ΔS or ΔfQ value greater than 1.0 JND indicates that the bird is able to perceive a difference between own species and heterospecific (cuckoo) egg colours [12,50]. In the statistical analyses, to remove problems with non-independence in these comparisons, JND values for each host or non-host species's egg were calculated relative to a randomly selected cuckoo egg sample of a particular cuckoo host-race, except for host and cuckoo eggs collected from the same nest, which were always paired. We used two-tailed one-sample *t*-tests to assess whether JNDs between parasite and host or non-host eggs were significantly different from 1.0.

(d) *Pigment composition*
We used a 5 per cent sulphuric acid in methanol extraction protocol [45] and a detection process using flow-injection electrospray ion-trap mass spectrometry, for the quantitative assessment of biliverdin IXα and protoporphyrin IX as their dimethyl esters, following the study of Igic *et al.* [42]. Each eggshell sample of measured area was dissolved in fresh 5 per cent sulphuric acid in methanol and steeped for 1–2 days (no longer than 2) before filtering through 1 ml barrier pipette tips (Axygen Biosciences) under pressure. The acidified methanolic filtrate was then extracted into dichloromethane/methanol/water (1 : 2 : 1 v/v/v) three times, recovering the lower phase each time, then washing the combined lower phases once in 10 per cent sodium chloride solution and twice with water, ensuring the pH of the final water wash was above 5. The organic solution was then evaporated to dryness under a stream of nitrogen and dissolved in 1 ml of methanol. A quantitative assessment of both biliverdin and protoporphyrin was conducted for these extracts by analysis on an ion-trap mass spectrometer. Samples were exposed to flow-injection analysis using an Agilent 1100 series capillary high-performance liquid chromatography, delivering 95 per cent methanol/0.1 per cent formic acid at a flow of 20 μl min^{-1} and coupled with an Agilent ion-trap mass spectrometer model SL with an electrospray ionization interface. Biliverdin IXα dimethyl ester and protoporphyrin IX dimethyl ester were then quantified simultaneously by multiple reaction monitoring, whereby protonated biliverdin IXα dimethyl ester was isolated at the molecular weight (m/z) of 611.4 and quantitated using the fragment at m/z 311.1, with fragments at m/z 209.1 and m/z 283.2 used as qualifier ions; and protonated protoporphyrin IX dimethyl ester was isolated at m/z 591.3 and quantitated using the fragment of m/z 513.3, with fragments at m/z 485.3 and m/z 445.3 used as qualifier ions. Biliverdin IXα dimethyl ester and protoporphyrin IX dimethyl ester standards were obtained from Frontier Scientific Inc. (Logan, UT, USA), and they gave a linear response over the range 8 fmol to 2.4 pmol.

Pigment concentrations were standardized by the surface area of each extracted eggshell sample because colourful pigments are predominantly found within the eggshell cuticle layer [51]. In the statistical analyses of these data, we used GLMMs to detect differences in natural logs of extracted biliverdin and protoporphyrin concentrations between host or non-host species relative to cuckoo host-races, and controlled for the extraction and the analysis-run date, egg sample and nest IDs, and collection year as random effects. The effect of year was removed from the model predicting biliverdin concentration owing to problems with negative

variance [52]. For the same reason [52], the effects of collection year and nest ID were removed from the model predicting protoporphyrin concentration.

To investigate relationships between pigment composition and background eggshell coloration, we first extracted residuals from GLMMs with log of pigment concentration (either biliverdin or protoporphyrin) as responses, with pigment analysis date as a fixed effect and egg ID as a random effect, to adjust for significant differences in pigment concentration across the different pigment analysis runs in the laboratory. We then constructed GLMMs with colour space PC variables or the combined excitation of MW and LW cones (brightness) as responses, with pigment concentration residuals as fixed effects and nest ID as a random effect.

(e) *Statistical analysis*
All statistical analyses were conducted using JMP v. 9 (SAS Institute, Cary, NC), EXCEL 2003 (Microsoft Corporation, Seattle, WA, USA), and STATVIEW v. 5.0.1 (SAS Institute). All data were checked for normality and heterogeneity of variance, and arcsine square-root transformations were used where appropriate. Sequential Bonferroni adjustments and Tukey HSD tests were used to avoid type I error rate inflation where multiple comparisons were made.

3. RESULTS
(a) *Spectral measurements and colour space analyses*
Physical measures of eggshell colour of parasite (electronic supplementary material, figure S1), host and non-host species detected global differences in avian colour space (figure 2; electronic supplementary material, figure S2), including both PC scores (PC1: $F_{9,73.71} = 15.98$, $p < 0.0001$; PC2: $F_{9,69.33} = 54.48$, $p < 0.0001$) and achromatic (brightness) quantum catch ($F_{9,73.05} = 6.41$, $p < 0.0001$). As predicted by the physical replication of colour hypothesis for local host-specific mimicry, both in Hungary and in Finland, each cuckoo host-race overlapped in some combination chromatic traits of eggshells only with their respective primary local hosts compared with local non-hosts (PC1). Although there was also extensive overlap in other chromatic traits (PC2) and brightness between each cuckoo host-race and its respective host species, these overlaps also included some of the local non-host species. In contrast, the Czech cuckoo host-race's eggs showed a better physical match with the eggs of both the Czech primary host and the Hungarian host species (both the great reed warbler), compared with the eggs of the Czech secondary host (the reed warbler, *Acrocephalus scirpaceus*), even though the cuckoo eggs were sourced from nests of the reed warbler in the Czech Republic.

(b) *Perceptual modelling*
Chromatic and achromatic JND contrasts (figure 3; electronic supplementary material, table S1) revealed that both the Hungarian cuckoo (figure 3a) and the Finnish cuckoo (figure 3b) host-races best matched their respective local host species. In contrast, comparisons of cuckoo eggs with local non-hosts and non-local hosts revealed JNDs not significantly below the discrimination threshold (i.e. 1.0 JND). As above, again, the Czech cuckoo (figure 3c) eggs sourced from the Czech secondary host (reed warbler) showed a better perceptual
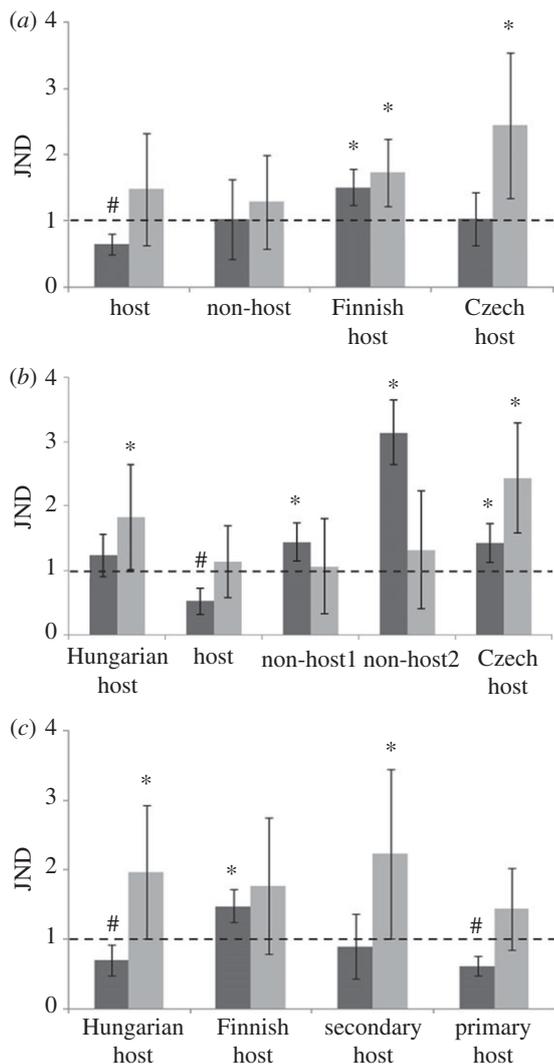
Figure 3. Avian-perceived differences, estimated as just noticeable differences (JND: mean ± 95% CI) for the chromatic (ΔS; dark grey bars) and achromatic (ΔfQ; light grey bars) components of colour matching between cuckoo and local host, non-local host and local non-host for the (*a*) Hungarian cuckoo host-race, (*b*) Finnish cuckoo host-race and (*c*) Czech cuckoo host-race. Dashed lines represent the discrimination threshold (1 JND) below which two egg colours are indistinguishable by the avian visual system. Asterisks indicate colour comparisons that are statistically significantly above discrimination threshold of 1.0 JND while octothorpes indicate colour comparisons that are statistically significantly below 1.0 JND (one-sampled *t*-test, $p < 0.05$).

match with the Czech primary host and Hungarian host species (both great reed warblers). Comparisons of JND values with different hosts between three cuckoo host-races demonstrated that the Hungarian cuckoo and Czech cuckoo were equally matched, but better than the Finnish cuckoo eggs when compared with both Hungarian host and Czech secondary host species (electronic supplementary material, table S2). In turn, the Finnish cuckoo was a better match to Finnish host eggs compared with the other two cuckoo host-races (electronic supplementary material, table S2).

(c) *Pigment composition*
Across all our sample comparisons (figure 4), we detected significant differences in both biliverdin ($F_{9,28.21} = 18.22$,

$p < 0.0001$; electronic supplementary material, figure S3*a*) and protoporphyrin ($F_{9,38.68} = 24.17$, $p < 0.0001$; electronic supplementary material, figure S3*b*) concentrations extracted from the different host species, non-host species and cuckoo host-races. As predicted by the chemical replication of mimetic colour hypothesis, biliverdin concentrations from cuckoo host-race eggs matched only their respective local host species eggs both in Hungary and in Finland, compared with local non-hosts. In turn, the Czech cuckoo eggs matched the biliverdin concentrations of both their primary hosts and the secondary hosts from whose nests these cuckoo eggs were sourced. Regarding protoporphyrin concentrations, in Hungary, cuckoo eggs again only matched their hosts, but not the local non-host species, whereas in Finland and in the Czech Republic, cuckoo eggs matched both hosts as well as some non-hosts and secondary hosts.

These differences in pigment composition overall reflect a separation of eggshells into two groups, based on colour (figure 4). Specifically, the immaculate blue eggs of the Finnish cuckoo and Finnish host (common redstart) showed, overall, higher biliverdin concentrations compared with eggs from all the other species and cuckoo host-races, including the local blue-egg-laying Finnish non-host1 (pied flycatcher, *Ficedula hypoleuca*; electronic supplementary material, figure S3*a*). In turn, the maculated, greenish-beige eggs of the Hungarian cuckoo and its host, the Czech cuckoo, and both its secondary and primary hosts, had on average higher protoporphyrin concentrations compared with the Finnish host, Finnish and Hungarian non-host species, and the Finnish cuckoo host-race (electronic supplementary material, figure S3*b*).

Both biliverdin and protoporphyrin concentrations affected perceived colour in the sensory modelling analysis (electronic supplementary material, table S3). Increased concentrations of both biliverdin ($F_{1,39.89} = 22.31$, $p < 0.0001$) and protoporphyrin ($F_{1,41.7} = 16.98$, $p = 0.0002$) resulted in a higher predicted excitation of LW and MW cones relative to UV and SW cones (PC1), while increased concentration of biliverdin resulted in a higher predicted excitation of SW and MW cones compared with UV and LW cones ($F_{1,41.62} = 36.22$, $p < 0.0001$), and increased protoporhyrin had the opposite effect ($F_{1,43.44} = 11.54$, $p = 0.0015$; PC2). Increased concentrations of both biliverdin ($F_{1,44.95} = 4.41$, $p = 0.04$) and protoporphyrin ($F_{1,44.77} = 7.95$, $p = 0.007$) were associated with decreased predicted excitation of MW and LW cones, used to model brightness perception.

4. DISCUSSION
We investigated the chemical basis of how different host races of obligate brood parasitic common cuckoos produce eggs to mimic their hosts' eggs and, in turn, manipulate the perceptual systems and cognitive decision rules of their hosts to accept costly foreign eggs within their clutch [53]. Host eggshell colour mimicry by specialist host-races of cuckoos, as perceived by UVS birds' eyes [12–14,21], is shown here to be associated with similar concentrations of the two key pigments predominantly involved in the egg-shell coloration throughout the Aves [40–42]. Still, the
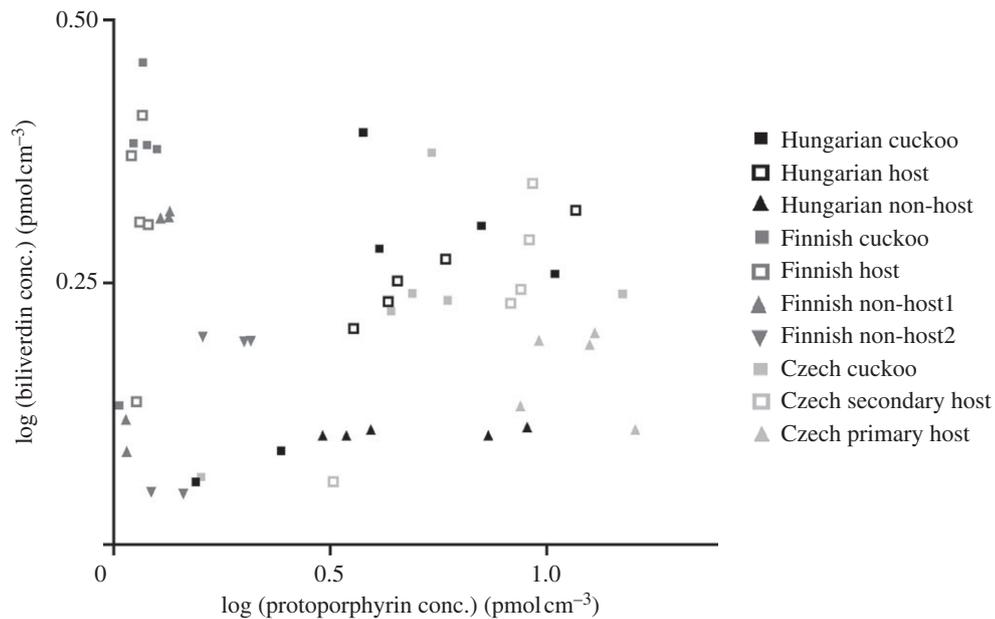
Figure 4. Concentrations of log(biliverdin IXα dimethyl ester) versus log(protoporphyrin IX dimethyl ester) for all cuckoo host-races, hosts and non-hosts from each locality.

potential roles of smell, taste and other chemical sensations that may be associated with different eggshell pigment compositions and concentrations in avian host–parasite discrimination, egg rejection and mimicry remain unknown [1].

Our study is based on data from recent field samples of unincubated eggshells (table 1), which help us to confirm the results of previous spectral and perceptual analyses based on, in part, museum specimens, because eggshell colour traits may shift with increasing time in storage [30,54,55]. Critically, the results of chemical and perceptual analyses here confirmed established patterns that host-races of the common cuckoo evolved differently coloured eggs to effectively mimic the avian-perceived egg coloration of their respective host species [12,13,21].

These data reveal a range of extents to which the different types of perceivable colour traits, including those associated with differences in brightness/achromaticity and colour/chromaticity, are matched by the cuckoo eggs with local host eggs across the three studied host-races. Specifically, in our perceptual analyses for two of the three geographically separate host–parasite systems, egg coloration was both predicted and shown to be best matched with host species' eggs compared with sympatric non-host species. These results are in line with the scenario that mimicry in the common cuckoo has adapted through its coevolutionary interactions with the respective hosts in Finland [12] and Hungary [25], and egg colour similarity has arisen not solely because of the shared habitat or microclimate at the respective local breeding sites [56]. These results suggest exciting new directions for future research not only into the chemical basis of eggshell colour mimicry, but also into the coevolution of sensory system tuning and cognitive decision rules across different host species of mimetic parasites [34,57].

Physical (figure 2) and perceivable egg colour matching (figures 1 and 3) and chemical concentrations (figure 4) between parasites' relative to local hosts' eggshells were closest for the Finnish cuckoo parasitizing common redstarts and the Hungarian cuckoo parasitizing

great reed warblers. In turn, the cuckoo eggs from the Czech Republic (sourced from reed warbler nests, locally the cuckoo's secondary host) were equally close in their pigment composition to both the Czech primary and secondary hosts (the great reed warbler and reed warbler, respectively) and the Hungarian host (the great reed warbler), yet they showed the best perceptual match only with the Czech primary host and Hungarian host eggs (both the great reed warbler) and not with the Czech secondary host eggs (the reed warbler). These Czech results correspond well with the known history of this site's particular host–parasite system, including the recently reported lack of evidence for local (i.e. population-specific) adaptation of cuckoo egg coloration across continental Europe in the same host species [58]. Specifically, reed warblers (secondary host) in the Czech study site (i) do not recognize adult cuckoos as specific enemies [43], (ii) reject non-mimetic foreign eggs at low rates [59] and (iii) reject cuckoo chicks at low rates [60], and these observations coupled with (iv) the coexistence of low levels of both egg and chick discrimination strategies are indicative of an early stage of parasite–host coevolution [61,62]. Indeed, the Czech reed warbler population has reportedly been frequently parasitized by cuckoos only since the second half of the twentieth century, following a dramatic population decline of the locally preferred and higher-foster-quality Czech great reed warbler host (discussed in Honza *et al.* [43]). Thus, the recent host switch from the great reed warbler to the reed warbler in our study Czech population may explain why cuckoo eggs found in the nests of the latter species are more similar to eggs of the former host, revealed in our analysis as an example of physical, perceptual and chemical evolutionary lag. In addition, our results on the great reed warbler–cuckoo relationship support the pattern that cuckoos adapt to their hosts at the metapopulation level, not only locally [58].

The spectrometric analyses performed here suggest that perceived colour metrics of host egg colour mimicry are best for the Finnish cuckoo host-race compared with

the other two investigated host-race systems. This confirms previous evidence of 'perfect colour matching' as judged by human observers for the Finnish common cuckoo race parasitizing common redstarts [63], a realistic sensory model of this host species perceptual physiology [28] (but see contrasting results from the UK population of this host and its parasite race [14]), and also the lack of any discrimination or ejection of naturally laid cuckoo eggs by redstarts [63]. Cavity nesting species such as the common redstart experience decreased costs of parasitism [64], so the efficient mimicry in the Finnish cuckoo host-race system is contrary to the expectation that egg mimicry should be less efficient in cuckoo/host systems where the costs of parasitism are lower [63]. Cavity nesting species may also be released from better mimicry if the chromatic similarity thresholds to detect perceptual differences among eggs are more permissive because of the reduction in available light in the nest hole [65,66]. Better egg colour matching between redstarts and their cuckoo host-race in Finland, therefore, may reflect a long coevolutionary relationship [16,18,19], including the recent discovery of the distinct genetic isolation of the Finnish redstart-cuckoo race from other cuckoo-races in Europe [16], whereby an evolutionarily stable strategy has evolved and rejection responses by hosts towards natural parasitism are seldom seen owing to a high risk of rejection errors [66]. An alternative hypothesis is that similarity may be due to chance or a recent egg colour adaptation where evolutionary lag or decreased cost of parasitism restricts the evolution of better host egg rejection abilities [63]. Contrary to this alternative, common redstarts do reject artificial, non-mimetic cuckoo eggs [63]. However, the evolution of eggshell coloration and the underlying pigment composition in brood parasite eggs may be driven not only by host discrimination of brood parasitic eggs, but also by shared environmental conditions [56]. Interacting effects of host and parasite maternal investment may also affect egg pigmentation and visible phenotype [10].

In parallel with our analyses of spectral and perceptual colour metrics, the chemical analyses revealed that in two of the three host–parasite systems, the concentrations of biliverdin and protoporphyrin in the cuckoo eggshells were more similar to local host eggshells compared with either local non-host or non-local host eggshells. Both biliverdin and protoporphyrin are involved in the vertebrate haeme metabolic pathway [67]. Thus, their ubiquitous involvement in regulatory processes may explain the shared phylogenetic availability and flexibility of incorporation of both these pigments into differently coloured eggshells both across and within diverse avian lineages [40–42]. This shared chemical basis of avian eggshell colour has now been revealed to include more similar pigment compositions between host and mimetic parasite eggs resulting in similar perceived phenotype.

Throughout this study, we provide correlative, and not experimental, evidence that host–parasite evolutionary history, and not shared habitat, is responsible for chemical and perceptual mimicry of host eggs by cuckoos. These novel results reveal that the concentrations of the metabolic products responsible for egg coloration throughout the Aves [40–42] are most similar between closely egg-mimetic cuckoos and their respective hosts. We suggest that eggshell pigment concentrations can be intraspecifically flexible, and in the case of the common cuckoo, this flexibility is used to generate mimicry of the physical and perceived egg coloration of its respective hosts by the genetically different host-races [15,16]. It still remains to be determined whether and how sex-chromosomal and autosomal control of the genetic basis of eggshell coloration is employed in generating cuckoo–host eggshell similarity [68,69]. The general evolutionary implication of these discoveries is that some novel morphological characters evolve from the modification of existing substrates and processes [70,71].

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Table S1: Mean chromatic and achromatic JNDs and t-statistics calculated from one-sample t-tests comparing matching of cuckoo host-race eggs to host and non-host eggs in relation to the discrimination threshold of 1 JND (▲ above and ▼ below). Asterices correspond to the levels of significance (* $0.05 > p > 0.01$, ** $0.01 > p > 0.001$ and *** $p < 0.001$).

Table S1

Comparison	Chromatic		Achromatic	
	Mean JND	*t*	Mean JND	*t*
Hungarian cuckoo vs Hungarian host	0.64▼	5.02***	1.47	1.20
Hungarian cuckoo vs Hungarian non-host	1.01	0.05	1.28	1.02
Hungarian cuckoo vs Finnish host	1.50▲	4.03**	1.72▲	3.14**
Hungarian cuckoo vs Czech secondary host	1.03	0.16	2.43▲	3.01*
Finnish cuckoo vs Hungarian host	1.24	1.62	1.84▲	2.22*
Finnish cuckoo vs Finnish host	0.53▼	4.94***	1.15	0.58
Finnish cuckoo vs Finnish non-host1	1.45▲	4.22*	1.07	0.28
Finnish cuckoo vs Finnish non-host2	3.15▲	11.90***	1.33	0.99
Finnish cuckoo vs Czech secondary host	1.44▲	3.37**	2.44▲	3.89**
Czech cuckoo vs Hungarian host	0.71▼	2.77*	1.97▲	2.19*
Czech cuckoo vs Finnish host	1.49▲	4.47***	1.77	1.72
Czech cuckoo vs Czech secondary host	0.91	0.45	2.23▲	2.34*
Czech cuckoo vs Czech primary host	0.62▼	5.57***	1.45	1.58

Table S2: T-statistics calculated from Bonferroni corrected *t*-tests comparing egg chromatic matching of two cuckoo host-races (first column) with a specific host species (second column). We computed the difference between a cuckoo host-race (e.g., Hungarian cuckoo) and a host (e.g., Hungarian host), the difference between another cuckoo host-race (e.g., Finnish cuckoo) and the same particular host (i.e., Hungarian host in the present example), and then tested the difference between these two values with a *t*-test. Asterices correspond to the level of significance (* $0.05 > p > 0.01$, ** $0.01 > p > 0.001$ and *** $p < 0.001$). Best matched cuckoo host-race eggs in the comparison are presented (last column). See Table 1 for denomination of groups.

Table S2

Cuchoo host-race comparison	Host species	*t*	Better match
Hungarian cuckoo - Finnish cuckoo	Hungarian host	−3.62**	Hungarian cuckoo
Hungarian cuckoo - Czech cuckoo	Hungarian host	−0.57	Equal
Finnish cuckoo - Czech cuckoo	Hungarian host	2.91**	Czech cuckoo
Hungarian cuckoo - Finnish cuckoo	Finnish host	6.21***	Finnish cuckoo
Hungarian cuckoo - Czech cuckoo	Finnish host	0.06	Equal
Finnish cuckoo - Czech cuckoo	Finnish host	−6.61***	Finnish cuckoo
Hungarian cuckoo - Finnish cuckoo	Czech secondary host	−1.91*	Hungarian cuckoo
Hungarian cuckoo - Czech cuckoo	Czech secondary host	0.45	Equal
Finnish cuckoo - Czech cuckoo	Czech secondary host	2.21*	Czech cuckoo
Hungarian cuckoo - Czech cuckoo	Czech primary host	0.58	Equal

STable 3: Slope estimates and associated standard errors (s.e.) for effects of biliverdin and protoporphyrin concentration on colour space PCs and combined MW and LW cone excitation used to model perceived brightness calculated from GLMMs. Adjusted R^2 values indicating explanatory power of a model are reported.

STable 3:

	PC1		PC2		Achromatic	
	Slope estimate	s.e.	Slope estimate	s.e.	Slope estimate	s.e.
Biliverdin	−6.56	1.39	6.27	1.04	−1.99	0.95
Protoporphyrin	−2.56	0.62	−1.58	0.46	−1.18	0.42
Adjusted R^2	0.96		0.95		0.70	

Figure S1: Average reflectance curves (± s.e. every 50 nm) for (*a*) Hungarian cuckoo, (*b*) Finnish cuckoo and (*c*) Czech cuckoo, host and non-host/secondary host eggshell background colours.

Figure S1

Figure S1

(c) Czech Republic
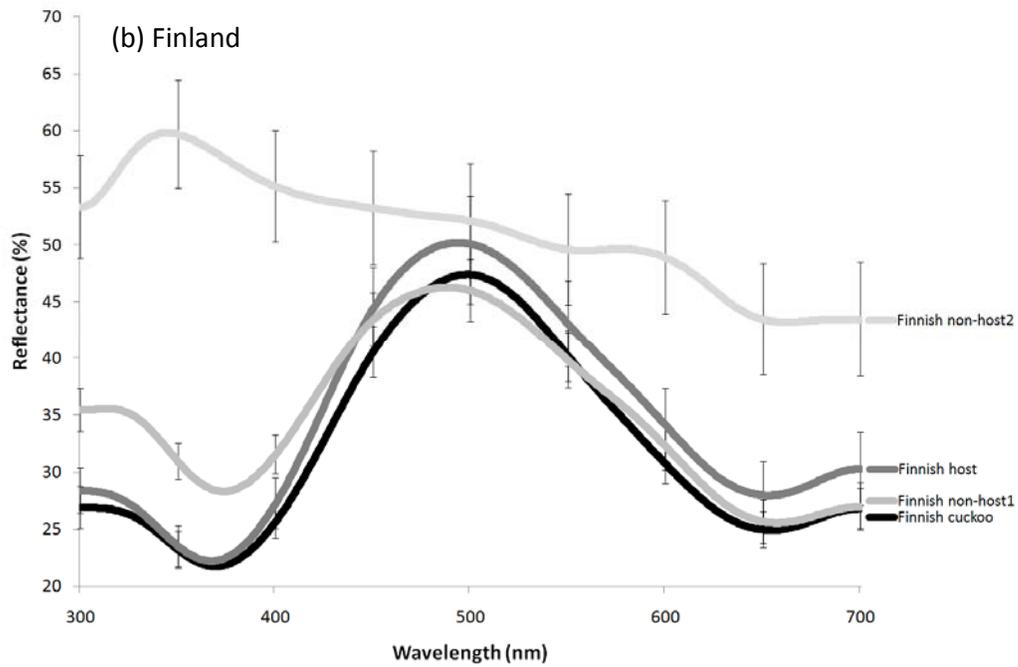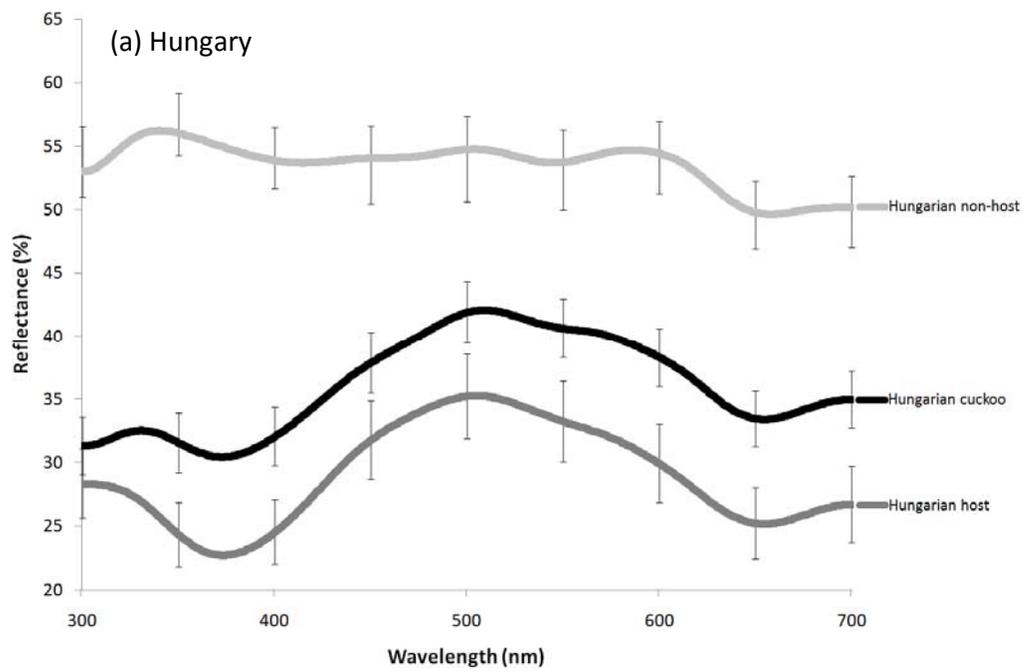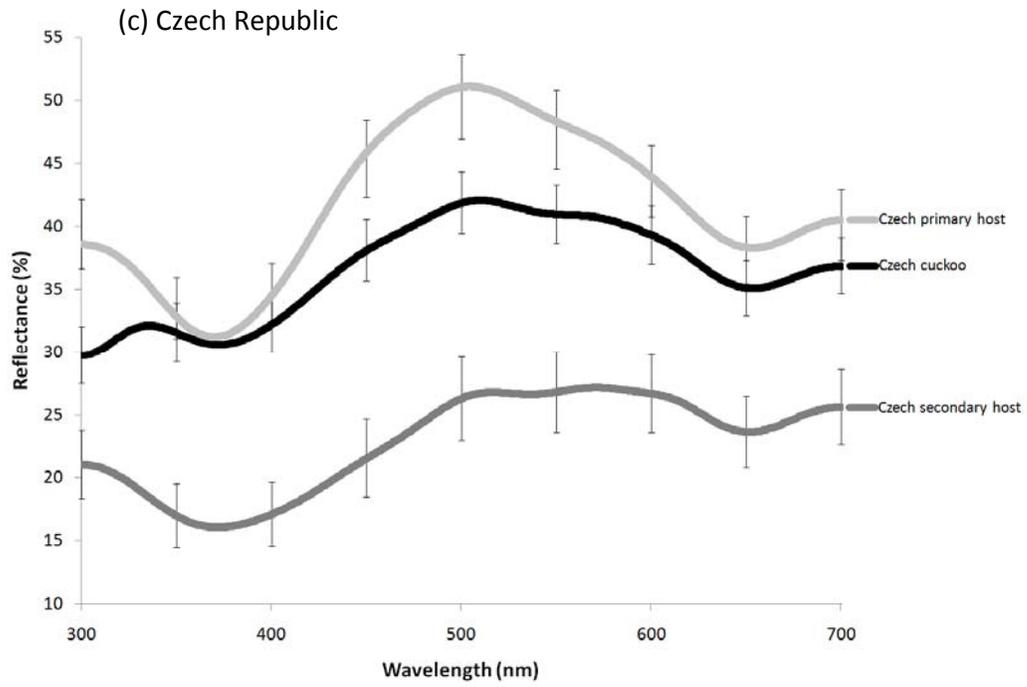
Figure S2: Principal component (PC) scores (mean ± s.e.) on (*a*) PC1 and (*b*) PC2 axes for avian colour space of cuckoo host-race, host and non-host egg background colours from three geographic locations. (*c*) combined excitation of MW and LW cones used to model percieved brightness (mean ± s.e.) of cuckoo host-race, host and non-hosts egg background colours from three geographic locations. Groups not connected by the same letter are significantly different in the general linear mixed model (Tukey HSD; *p* < 0.05). Dark bars = Hungarian samples, medium bars = Finnish samples and light bars = Czech samples.

Figure S2:

(a)
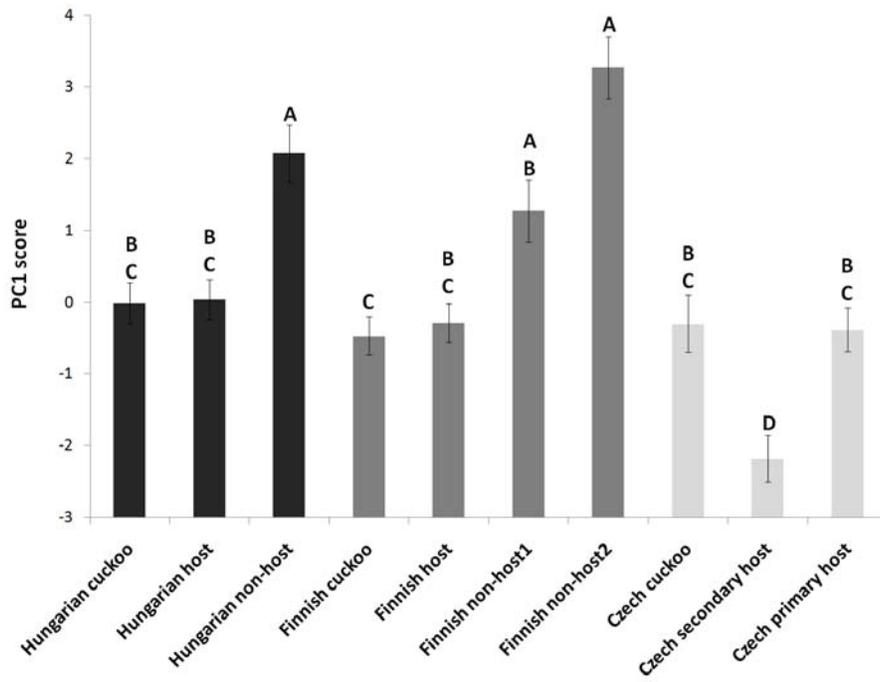
(b)
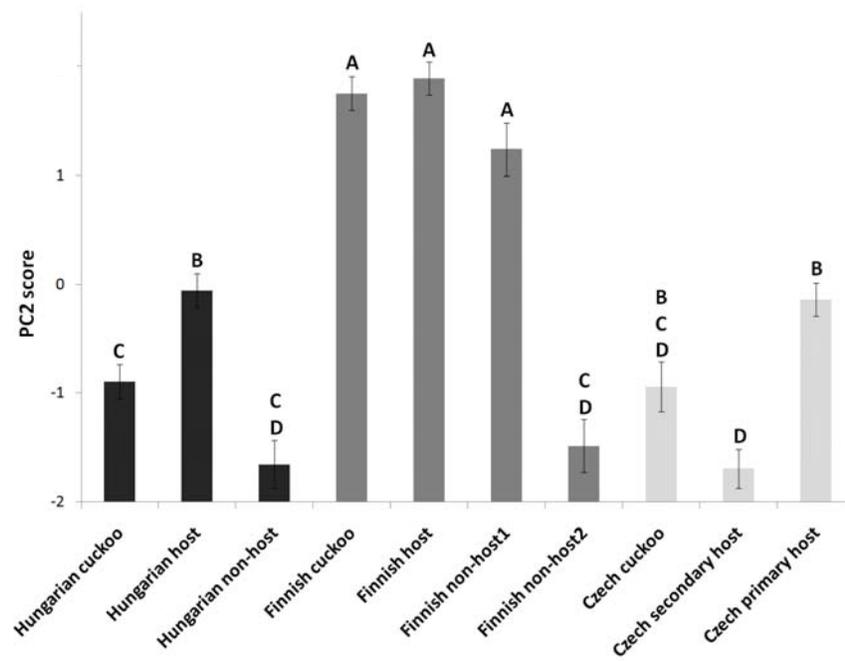
Figure S2:
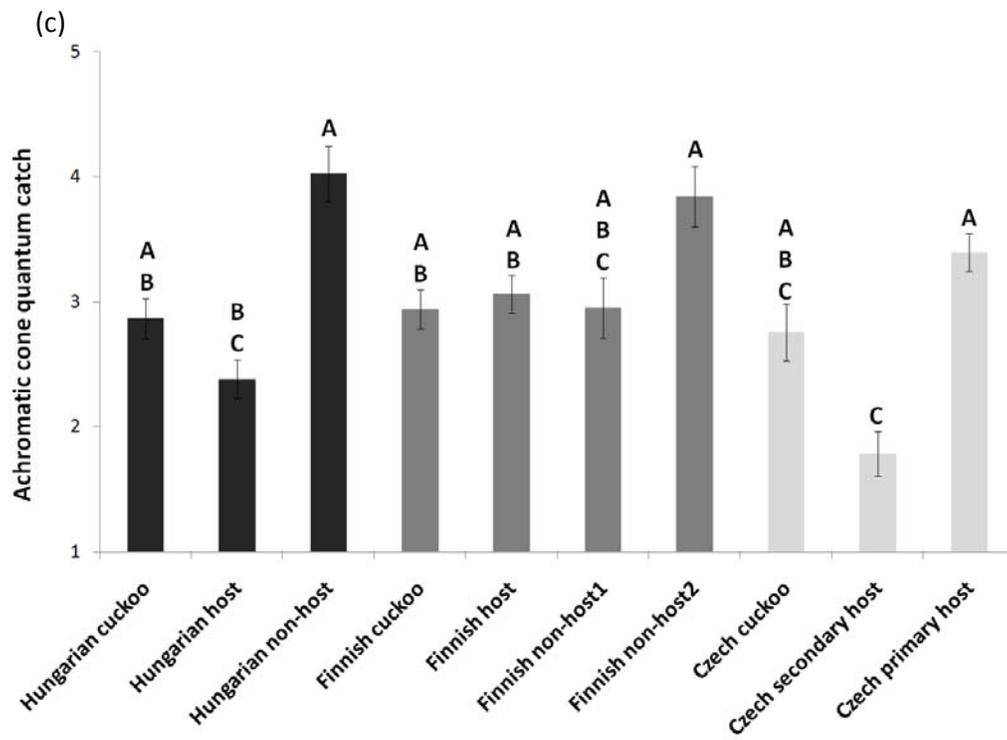
Figure S3: Concentrations (mean ± s.e.) of (*a*) biliverdin IXα dimethyl ester and (*b*) protoporphyrin IX dimethyl ester of cuckoo host-races, hosts and non-hosts hosts from each locality. Groups not connected by the same letter are significantly different in the general linear mixed model (Tukey HSD; *p* < 0.05). Dark bars = Hungarian samples, medium bars = Finnish samples and light bars = Czech samples.

Figure S3:

(a)

(b)