Prey animals that possess chemical defences often advertise their unprofitability to predators by a distinctive and conspicuous visual appearance known as aposematism. However, not all chemically defended species are conspicuous, and some are nearly cryptic. Differences in predator community composition and predator behaviour may explain varied levels of prey conspicuousness. We tested this hypothesis by measuring dietary wariness and learning behaviour of day-old chickens (*Gallus gallus domesticus*) from four strains of laying hens that have been selected for different levels of egg productivity. We used these strains as model predators to test if predators that vary in the trade-offs associated with foraging behaviour, cause differential survival of chemically defended prey with conspicuous signals. We show that strains differ in how they learn about chemically defended prey, which result in significant differences in prey survival. The selection pressures imposed by different types of predator could explain whether chemically defended prey evolve varied levels of conspicuousness. Predators' initial wariness of novel prey was not related to learning at the strain or individual level, but predator wariness increased after exposure to chemical defences. Our study provides support for the hypothesis that the evolution of prey defences depends on variation between ecological communities in predator learning behaviour and experience.

Keywords: Aposematism, conspicuousness, dietary wariness, learning, selection.

Prey animals often advertise their chemical defences to predators by a distinctive and conspicuous visual appearance known as aposematic signalling (Wallace, 1889). Aposematism, which is typified by the red and black colouration of ladybirds (Majerus & Kearns, 1989) and the black and yellow stripes of cinnabar moth caterpillars (Aplin, Benn, & Rothschild, 1968), accelerates predator avoidance learning (Gittleman & Harvey, 1980; Roper & Wistow, 1986), and enhances predator memory of prey best avoided (Roper & Redston, 1987). Stronger, more visible signals facilitate faster avoidance learning compared to weaker signals, and can make the difference between predators learning or not learning to avoid aposematic prey (Alatalo & Mappes, 1996; Lindstrom, 1999; Mappes & Alatalo, 1997; Roper & Redston, 1987). However, not all defended prey advertise their defences with conspicuous signals (Arbuckle & Speed, 2015; Lindstedt, Huttunen, Kakko, & Mappes, 2011). Many chemically defended species have variable colour patterns along their distribution range, for example polytypic poison frogs, (Willink, García-Rodríguez, Bolanos, & Proehl, 2014), and polymorphic ladybirds and moths (Majerus & Kearns, 1989; Nokelainen, Valkonen, Lindstedt, & Mappes, 2014). What causes some defended species to be distinctive and conspicuous and others inconspicuous?

This question has been explored theoretically, comparatively, and empirically (Endler & Mappes, 2004; Ratcliffe & Nydam, 2008; Valkonen et al., 2012). Theoretical models predict that differences in predator perception and/or learning behaviour can explain whether prey evolve aposematism rather than crypsis (Endler, 1988), aposematic polymorphisms (Mallet, 2001; Mallet & Joron, 1999; Mallet & Singer, 1987), or ‘weak’ aposematic signals (Endler & Mappes, 2004). Comparative analyses have revealed that tiger moths are more likely to deploy conspicuous visual

46 warning signals when birds are their main predators, and ultrasonic clicks when bats
47 are more prevalent (Ratcliffe & Nydam, 2008). Predators with different sensory
48 capacities have also been implicated in how aposematic signal size varies in Japanese
49 fire-bellied newts (*Cynops pyrrhogaste*; Mochida, 2011). These theoretical and
50 comparative findings are supported by a number of empirical studies. For example,
51 Valkonen et al. (2012), in an experiment with warningly and non-warningly coloured
52 artificial snakes, found that in habitats dominated by specialist predators, artificial
53 snakes with conspicuous warning signals are attacked more than inconspicuous
54 snakes; in habitats dominated by generalist predators, the inconspicuous snakes were
55 attacked more frequently than the conspicuous. Therefore, specialist predators may
56 select for reduced conspicuousness, whereas generalist predators may select for
57 conspicuous warning signals. Differences in the age/experience of predators can
58 explain why aposematic signals are more prevalent in some seasons compared to
59 others (Mappes, Kokko, Ojala, & Lindström, 2014). Furthermore the predominant
60 predator species in a habitat may have a greater influence on the maintenance of
61 aposematic signal polymorphisms than less prevalent predator species (Nokelainen et
62 al., 2014). These studies provide evidence that different predator behaviours can
63 affect the fitness of aposematic signals and how they evolve.

64 Guildford and Dawkins (1991) proposed that differences in how a predator
65 detects, discriminates, learns, and remembers a signal can represent a powerful
66 selective force in signal design. Most research on predator behaviour and warning
67 signals has focused on a single aspect of the predator's 'receiver psychology'
68 (Guilford & Dawkins, 1991), such as detectability (Siddiqi, Cronin, Loew, Vorobyev,
69 & Summers, 2004), discrimination (Skelhorn & Rowe, 2006a), or avoidance learning
70 (Ihalainen, Lindström, & Mappes, 2007). However, the interaction between these

71 different behaviours can affect selection on prey defences (Skelhorn, Halpin, & Rowe,
72 2016). A predator's ability to learn about prey types may be related to its initial
73 reaction towards that prey (Schuler & Roper, 1992), and how predators learn can
74 affect how they remember prey (Ihalainen et al., 2007; Roper & Redston, 1987), and
75 how they generalise their knowledge about those prey (Gamberale-Stille & Tullberg,
76 1999). In this study we focused on two candidate predator behaviours that may differ
77 across individuals and species, and affect how warning signals evolve: differences in
78 the initial responses towards novel and/or aposematic prey, and differences in the
79 ability to learn to avoid aposematic prey (Sherratt, 2002).

80 Differences in the initial responses towards novel and/or aposematic prey can
81 be characterised by a short-lived avoidance of novel/conspicuous prey (neophobia), or
82 longer-term refusal to eat novel/conspicuous prey (dietary conservatism: Exnerová et
83 al., 2015; Exnerová, Svádová, Fučíková, Drent, & Štys, 2010; Marples & Kelly, 1999;
84 Marples, Quinlan, Thomas, & Kelly, 1998). Neophobia and dietary conservatism are
85 collectively referred to as dietary wariness. Predators that are wary of - and avoid
86 attacking - novel and/or conspicuous prey may allow conspicuous signals to increase
87 in abundance (Marples & Mappes, 2011; Richards et al., 2014; Thomas, Marples,
88 Cuthill, Takahashi, & Gibson, 2003) to the extent that learned predator avoidance
89 favours aposematism (Lee, Marples, & Speed, 2010; Mappes, Marples, & Endler,
90 2005; Puurtinen & Kaitala, 2006). It has been argued that any selective benefit to
91 conspicuous prey of being avoided by wary predators is transient at best, (Mallet &
92 Singer, 1987), because of variability in predator life span and wariness (Lee et al.,
93 2010). However, varied levels of dietary wariness may promote the spatial mosaics of
94 prey phenotypes that are seen in nature, especially if dietary wariness combines with

95 differences in predator avoidance learning (Lee et al., 2010; Sherratt, 2002). This
96 prediction warrants empirical investigation.

97 Differences in predator avoidance learning are known to emerge because of
98 differences in predator personality traits (Exnerová et al., 2010), developmental
99 conditions (Bloxham, Bateson, Bedford, Brilot, & Nettle, 2014), nutritional state
100 (Barnett, Bateson, & Rowe, 2007), and the complexity of the prey community in
101 which the predator forages (Ihalainen, Rowland, Speed, Ruxton, & Mappes, 2012).
102 For a comprehensive review of the factors that affect learning see: Skelhorn et al.,
103 (2016). Variability of predator learning has been found to affect the fitness of
104 aposematic prey and select for signal uniformity (Halpin, Skelhorn, & Rowe, 2012;
105 Skelhorn and Rowe, 2007b). Differences in predator learning could also explain the
106 varied levels of warning signal conspicuousness, but this remains an open
107 experimental question (Endler & Mappes, 2004).

108 Empirical studies that examine the links between predator wariness and
109 learning are scarce (Exnerová et al., 2010; Sillen-Tullberg, 1985).
110 Neophobia/wariness may be unrelated to learning processes (Braveman & Jarvis,
111 1978). However, a warning signal to which predators are reluctant to respond by
112 initiating an attack can theoretically induce faster avoidance learning and differential
113 selection (Guilford & Dawkins, 1991; Rowe & Guilford, 1999; Sherratt, 2002). In a
114 study with fast versus slow-exploring predators, Exnerová et al (2010) found that
115 slow birds hesitated longer to attack novel aposematic prey, and subsequently took
116 fewer trials to learn to avoid the same prey. However, the selection pressures imposed
117 by these different types of predator did not result in differential mortality of
118 aposematic prey. What remains unclear is whether differences in individual or species
119 wariness combine with learning to produce differential selection pressures on prey,

120 and if this can explain whether chemically defended prey evolve varied levels of
121 conspicuousness.

122 To resolve this issue, we designed an experiment in which day-old domestic
123 chicks (*Gallus gallus domesticus*) acted as model predators, as they have in much of
124 the empirical research into wariness and the evolutionary dynamics of warning signal
125 evolution (Marples et al., 2007; Roper & Redston, 1987; Roper & Wistow, 1986;
126 Rowe & Skelhorn, 2005). There are intra- and inter-strain differences in how chickens
127 react to and learn about novel and/or aposematic prey (Hauglund, Hagen, & Lampe,
128 2006; Jones, 1986). We propose that intra- and inter-strain differences might be useful
129 for the study of warning signal evolution, because they could be a simple way of
130 simulating species and individual differences that are also observed in wild predators
131 (Adamová-Ježová, Hospodková, Fuchsová, Štys, & Exnerová,.2016; Marples &
132 Kelly, 1999; Marples, Roper, & Harper, 1998). Intra- and inter-strain differences can
133 also provide information about feeding and learning in a domestic crop animal of
134 major importance, and also about the effects of selection on these behaviours (Schütz,
135 Forkman, & Jensen, 2001).

136 We studied four laying strains of chickens that have been selectively bred for
137 different levels of egg production and growth. Selection on these traits in modern
138 poultry is linked to reduced fearful behaviours, compared to their wild-type ancestors
139 - the red junglefowl (Campler, Jöngren, & Jensen, 2009; Schütz et al., 2001). When
140 populations of red junglefowl are selectively bred for a 'domesticated phenotype',
141 traits similar to modern chickens emerge after only a few generations, e.g., larger
142 body size, larger eggs and offspring (Agnvall, Ali, Olby, & Jensen, 2014), and
143 increased boldness in novel object tests (Agnvall, Katajamaa, Altimiras, & Jensen,
144 2015). Based on this evidence for reduced fearfulness in high productivity lines, we

predicted that (1) high productivity strains would exhibit lower dietary wariness (consume novel prey sooner) than lower production domestic strains.

High productivity strains also exhibit reduced contrafreeloading i.e. prefer to choose easily accessible food over food that requires work (Schütz & Jensen, 2001). Reduced contrafreeloading results in individuals acquiring less information during foraging (Lindqvist, Schütz, & Jensen, 2002). From this we predicted that (2) high production strains would be less discriminating between foods and therefore acquire less information during learning (compared to strains that have lower productivity and growth), and attack a higher proportion of chemically defended prey in learning trial eight. Based on the findings of Exnérova et al (2010) we also predicted that (3) strains with higher initial dietary wariness would attack a lower proportion of chemically defended prey in learning trial eight compared to strains with lower dietary wariness; and (4) individuals within strains with higher initial dietary wariness would attack a lower proportion of chemically defended crumbs in learning trial eight compared to individuals with lower dietary wariness. Our final prediction was that (5) experience of chemically-defended prey would increase dietary wariness towards a new novel food, previously not associated with chemical defences, in all strains (following Marples et al 2007).

The strains we studied were (i) Rhode Island Reds, a strain that has not undergone selection for high production traits and lays about 260 eggs per year; (ii) Black Rocks, a hybrid strain selected from Rhode Island Red (cockerels) and Barred Plymouth Rock (hens), that produces about 280 eggs per year; (iii) ISA Warren, a hybrid strain that contains genetics from a wide range of breeds but is thought to originate from crossing Rhode Island Reds with other pure breeds, is selected mainly for egg production and feed efficiency, yielding 320 eggs per year (Hendrix-

Genetics); and (iv) Hy-Line, a common breed used for egg production, developed from breeding Rhode Island Red and Leghorns, and selected for high food conversion efficiency, laying about 350 eggs per year (Hyline-International; Schütz and Jensen, 2001).

Methods

Subjects and housing

A total of 180 day old male domestic chicks (*Gallus gallus domesticus*) of four laying strains: Hy-Line (*N*=60), ISA Warren (*N*=40), Black Rock (*N*=50), and Rhode Island Red (*N*=30) participated in this study. The different strains were tested sequentially during January and February 2011, except for Rhode Island Red and ISA Warren chicks, which were tested in parallel. Each strain was from a single batch.

All chicks were marked with individual identifying colour codes on the top of their heads with non-toxic Sharpie™ marker pens. Markings did not result in any aggressive behaviour between individuals (Rowland, personal observation). All chicks were housed at the Institute of Biodiversity, Animal Health and Comparative Medicine at the University of Glasgow. All staff that trained, observed, and performed husbandry on the birds wore white lab coats at all times.

Cages measured 100x50x50cm with 10 chicks per cage. Chicks were subject to a 14:10h light:dark cycle and the lighting had no UV component. Each cage was heated to 27^{o}C, following guidelines to the operation of the Animal (Scientific Procedures) Act 1986 (2009), using either one Interbrooda standard (40cm x 60cm) or two Interbrooda mini (40cm x 40cm). These brooders, also known as ‘electric hens’, consist of an electrically heated square or rectangular plate that stands on four

194 adjustable legs, enabling the adjustment of height and temperature as the chicks grow.
195 The laboratory was held at a constant temperature of 24^{o}C. Temperatures beneath the
196 brooders and the ambient room temperature were monitored and recorded daily.
197 Water was provided *ad libitum* in two white one-litre drinkers in each cage. Brown
198 chick starter crumbs were also provided *ad libitum* in each cage in two beige ceramic
199 food bowls. We placed a clear plastic cylinder in each bowl, which reduced the
200 tendency of the chicks to sit in the food. The cages were lined with brown paper cage
201 liners, which were replaced daily. During training and experimenting, periods of food
202 restriction were necessary to motivate chicks to forage. During all periods of
203 restriction, chicks had access to water but not food. All restriction periods were in
204 accordance with UK Home Office regulations and guidelines, and were no longer
205 than one hour.

206

207 *Ethical note*

208 This study was conducted under UK Home Office Licence 60/4068. At the end of the
209 experiment because the chicks were all cockerels and could not be rehomed, Hy-Line,
210 Black Rock, and ISA Warren chicks were euthanised by Home Office schedule one
211 methods (cervical dislocation), following the Association for the Study of Animal
212 Behaviour's Guidelines for the treatment of animals in behavioural research and
213 teaching (2012). However Rhode Island Reds were rehomed to local smallholdings.

214

215 *Experimental food*

216 Palatable and chemically-defended prey were produced by spraying 150 g of chick
217 starter crumbs with either 100 ml of water or a 3% mixture of chloroquine phosphate

(following the methods of Rowland, Hoogesteger, Ruxton, Speed, & Mappes, 2010). When chick starter crumbs are coated with quinine/chloroquine at concentrations ranging from 1-6%, chicks learn to avoid quinine-coated crumbs and to forage on palatable crumbs (Rowland et al. 2010; Skelhorn & Rowe, 2006b), and they eat significantly fewer of the quinine-coated crumbs that they attack than the palatable crumbs they attack (Skelhorn & Rowe 2006a).

Crumbs were coloured either black, green, orange, or blue by spraying 150 g of the crumbs with 8 ml of Supercook black food dye added to 82 ml of tap water or 0.5 ml of Sugarflair spruce green, tangerine / apricot, or baby blue food dye added to 82 ml of tap water. These concentrations produced similar levels of luminance in the crumbs (we measured the spectral properties of the crumbs with an Ocean Optics spectrophotometer). All crumbs were allowed to dry for 24 h before sieving them to select crumbs of a similar size for the experiment.

Pre-training (day 1)

On arrival at the laboratory chicks were allowed to acclimatise for three hours, after which food was removed from the cages in a staggered order so that food restriction in any one cage did not last more than an hour during training, thereby standardising hunger levels between individuals. After approximately 30 minutes of food restriction, chicks commenced pre-training to build familiarity with the arena and foraging alone. Without such training, chicks placed in the arena alone become distressed, calling loudly and refusing to eat (Rowland, personal observation).

One person conducted pre-training of the chicks using three experimental cages simultaneously. These cages were identical to the home cages, except that a

mesh divider separated a buddy arena, measuring 20cm x 50cm x 50cm, from an experimental arena of 100cm x 50cm x 50cm (see Skelhorn & Rowe, 2006b for a schematic). There was no brooder, and the floor was covered with the white backing paper of sticky-backed plastic (a waxy paper imprinted with a faint black grid whose intersections were 2.5cm apart). All chicks participated in six four-minute pre-training sessions, during which they were required to forage on un-dyed chick starter crumbs that were scattered on the floor of the experimental arena. In trials one and two, chicks were placed in the experimental arena in groups of three; in trials three and four, chicks were placed in the arena in pairs. In trials five and six, lone chicks were placed in the arena (but in the presence of two buddies in the buddy arena). Buddy chicks reduce any potential distress among lone experimental chicks (Skelhorn & Rowe, 2006b). Buddy chicks never acted as experimental subjects in the neophobia or learning trials, and only provided company for the experimental chick. The buddies had free access to water but not food throughout their accompaniment of the experimental chick, so that the experimental chick was not distracted by familiar food in the buddy arena. Buddy chicks had free access to food in their home cages. We changed the buddy chicks for new buddies every three trials or between 30-60 minutes, whichever came sooner, so that restriction never exceeded the guidelines to the operation of the Animal (Scientific Procedures) Act 1986 (2009). By the end of pre-training, all experimental chicks were eating brown starter crumbs from the arena without any signs of distress.

First neophobia and dietary wariness assay (day 2)

The day after pre-training each chick was screened for its level of neophobia and dietary wariness in the same cages used for pre-training. We defined the duration of

neophobia as the latency to begin pecking at a novel food (following Marples & Kelly, 1999). Marples et al. (2007) define dietary wariness as the time an individual takes to consistently eat novel food. However, the exact criterion for what constitutes eating food consistently varies between experiments, e.g., consumption of novel-coloured food on three successive trials (Marples et al., 1998); time to eat a total of ten novel food items (experiment one Marples et al., 2007); time to eat three novel food items (experiment two of Marples et al., 2007); more than five consecutive pecks at novel food (Camín, Martín-Albarracín, Jefferies, & Marone, 2015). The methods for testing dietary wariness also vary depending on the species assayed (Marples & Kelly, 1999), and for birds the tests also vary from a choice between a 50:50 ratio of familiar and novel food (Marples et al., 1998; McMahon 2013; McMahon, Conboy, O'Byrne-White, Thomas, & Marples, 2014) to 99 familiar and one novel prey (Marples & Mappes 2011), to a small pile of novel food (Marples et al., 2007).

We followed the methods of Marples et al (2007 experiment one) and defined dietary wariness as the time to eat ten pieces of novel food, but we also measured the time to eat one piece of novel food (because our preliminary work indicated that once a bird had eaten one piece it went on to consume at least two more pieces of food in succession; Rowland 2010). The overall duration of dietary wariness therefore incorporates both the duration of neophobic avoidance plus the duration of avoidance due to dietary conservatism (Marples et al., 2007). Dietary wariness is therefore a biologically meaningful measurement of the time taken to incorporate a novel food into the diet and of the time during which the prey is somewhat protected by its novelty (Marples et al., 2007).

Chicks were placed into the main part of the arena, and two buddy chicks were placed in the small buddy area. Following the methods of Marples et al (2007,

292 experiment one), each experimental chick was offered a small pile of edible black
293 chick crumbs (black being a novel colour of food for these chicks). Each screening
294 session lasted for three minutes, during which time we recorded the number of crumbs
295 pecked and eaten. If the chick did not consume 10 crumbs in the first three minutes it
296 was removed from the cage and, following an interval of approximately 30 minutes,
297 re-tested until it had eaten 10 crumbs in total (which took a maximum of four trials or
298 720s). To ensure that chicks were not avoiding food simply because they had not
299 noticed it, we picked up any chick that had not pecked at the food after two minutes
300 and placed it beside the food (following Marples et al., 2007).

301

302 *Avoidance learning (days 3-6)*

303 After the neophobia and dietary wariness assay, experimental chicks (Hy-Line *N*=36,
304 ISA Warren *N*=24, Black Rock *N*=35, and Rhode Island Red *N*=20) participated in
305 the study. The remaining chicks acted as buddies. Experimental chicks were randomly
306 assigned into one of two treatments – either orange defended and green palatable, or
307 green defended and orange palatable (both orange and green were novel colours).
308 After 30-60 minutes of food restriction, a chick was placed in the experimental arena
309 alone (though in the presence of two buddies) where it encountered 20 palatable and
310 20 defended crumbs. We placed crumbs singly in the faint black grid (intersections
311 every 2.5 cm) on the floor of the experimental arena. We generated randomized maps
312 prior to the experiment to determine the position of each crumb. All prey were
313 presented on the same white background used in pre-training and wariness assays, so
314 that the chemically defended prey were no more conspicuous than the edible prey.

We recorded the identity and order of crumbs attacked, and whether the crumb was pecked or eaten. Chicks were required to peck or eat 16 crumbs to end a trial. All chicks received eight of these trials in total: two each on days 3, 4, 5 and 6. Therefore, this experimental design tested how learning varies between different strains of model predators that were maintained under the same conditions.

Second neophobia and dietary wariness assay (day 7)

After completing eight learning trials, all of the experimental chicks were tested for their response to a new novel colour of food (methods were the same as on day 1). Blue was chosen as the novel colour because it was clearly distinct from orange and green. Chicks were offered a small pile of blue food, which was novel for all the treatment groups. Each test session lasted for three minutes, and chicks were tested for three sessions or until they ate 10 crumbs, whichever occurred sooner. We chose to end the tests after three sessions because chicks that had not eaten any prey by the end of three sessions (540s) continued to avoid the novel food for so long that we would not have been able to complete testing all the birds on the same day. If chicks had not eaten any prey by the end of the three sessions they were assigned the maximum time (540s). The latency to peck at the food and the latency to eat one and 10 crumbs were recorded.

Statistics

The methods used to test our five hypotheses are outlined below. All of the statistical tests were conducted in STATA (StataCorp, 2011).

(1) The high-egg-productivity strains would exhibit lower dietary wariness than lower-productivity strains. To test this we log transformed the time to eat the first and tenth novel food item in the first neophobia and dietary wariness assay (from day 2), and tested for differences between strains using a linear regression model with strain fitted as a categorical variable, and mean egg production (described in the introduction as the mean number of eggs produced per year) fitted as a continuous variable.

(2) That high production strains would attack a higher proportion of chemically defended crumbs in learning trial eight compared to low productivity strains. To test this we used a least squares regression model on the logit (i.e. logarithm of the odds, used to linearise the relationships and stabilise the variance) of the proportion of chemically-defended crumbs attacked in trial eight of the learning experiment. We used a robust standard errors structure to allow for heterogeneity of variance (using the Huber-White sandwich estimator).

(3) That strains with higher initial dietary wariness would attack a lower proportion of chemically defended crumbs in learning trial eight compared to strains with lower dietary wariness. To test this we fitted the mean strain DC score (the mean of the log time that each strain took to attack the first and the 10^{th} novel food item in the first neophobia and dietary wariness assay on day 2), and egg productivity both as continuous variables in the least squares regression model for prediction 2, with robust standard errors allowing for intra-strain-correlation.

(4) That individuals within strains with higher initial dietary wariness would attack a lower proportion of chemically defended crumbs in learning trial eight compared to individuals with lower dietary wariness (wary individuals would have lower asymptotic levels of attack). To test this we fitted individual latency to attack the first

and 10^{th} novel food item in the first neophobia and dietary wariness assay (from day 2) with strain as a categorical factor.

(5) That experience of chemically-defended prey would increase dietary wariness towards a new novel food, previously not associated with chemical defences, in all strains. To test this we used a random effects interval regression model that allows for the lack of independence of the two observations for the same individual, and tested if dietary wariness changed between the first novel food choice test on day 2 and the novel food choice test after the learning experiment on day 7.

To test whether the proportion of chemically-defended prey attacked in the eighth trial could be explained by differences in prey handling throughout learning we constructed a rejection index—the proportion of chemically-defended crumbs pecked in the first seven trials that were rejected (i.e. not eaten).

Results

Strain differences in dietary wariness

In the first neophobia and dietary wariness test, we found some support for our hypothesis that high production strains would exhibit lower dietary wariness (measured as the latency to eat the first and the 10^{th} novel food item) than lower productivity strains. There was a significant difference between the strains in their time to eat the first novel food item (Figure 1 grey bars; $F_{2, 110} = 6.26$, $P = 0.003$), and their time to eat 10 pieces of novel food (Figure S1. $F_{(3,110)}=10.89$, $P < 0.001$). Strains with higher annual egg productivity attacked the 1^{st} novel food item sooner than strains with lower productivity ($t = -3.11$, $P = 0.002$).

386 The strain selected for highest egg production (Hy-Line, 350 eggs per year)
387 was composed of individuals that all exhibited short latencies to start consuming
388 novel food (see table 1 and cluster analysis methods in the Appendix). ISA Warren
389 (320 eggs per year) and Black Rock (280 eggs per year) had 71% and 86% of
390 individuals that showed low wariness, respectively (shorter latency to consume novel
391 prey). The strain with lowest annual egg productivity - Rhode Island Red (260 eggs
392 per year) - had the lowest proportion (60%) of individuals with low wariness.

393 The strain selected for highest egg production (Hy-Line) was significantly less
394 wary than Black Rock in their time to eat the first and 10^{th} novel food item (1^{st} t =
395 3.11, $P = 0.002$, 10^{th} t = 5.03, $P < 0.001$), and ISA Warren (1^{st} t = 4.23, $P < 0.001$,
396 10^{th} t = 2.73, $P = 0.006$) and Rhode Island Red (1^{st} t = 5.14, $P < 0.001$, 10^{th} t = 3.335,
397 $P = 0.001$). The residual effect of strain that could not be explained by mean annual
398 egg production accounted for 23% of the variation in the time to eat the first novel
399 food item.

400

401 *Strain differences in learning*

402 During the learning trials, chicks that received orange-defended crumbs did not learn
403 differently to chicks that received green-defended crumbs ($t = -0.44$, $P = 0.660$), so
404 we combined the attack data from the two treatment groups in learning trial eight in
405 the analysis. All four strains of chicken learned to attack fewer chemically-defended
406 crumbs by the end of the avoidance learning experiment (figure 2; $F_{4, 110} = 82.52$, $P <$
407 0 .0001), because they attacked significantly fewer defended crumbs in learning trial
408 eight compared with learning trial one (Hy-Line: $t = -5.52$, $P < 0.001$; ISA Warren: t
409 = -3.43, $P = 0.001$; Black Rock: $t = -15.28$, $p < 0.001$; Rhode Island Red: $t = -7.38$, P

410 < 0.001). However, the four strains differed in their level of avoidance learning
411 (calculated as the proportion of chemically-defended prey attacked in learning trial
412 eight: figure 2; $F_{3, 110} = 14.10$, $P < 0.0001$). The strain selected for highest egg
413 production (Hy-Line) did have a higher asymptotic attack level than the strain with
414 lowest productivity (Rhode Island Red: $t = -4.31$, $P < 0.001$), but did not have a higher
415 asymptotic attack level than two other strains (ISA Warren: $t = 0.92$, $P = 0.359$, and
416 Black Rock: $t = -0.89$, $P = 0.374$).

417

418 *The association between dietary wariness and learning - strains*

419 The strain differences the proportion of chemically-defended prey attacked in learning
420 trial eight were not explained by strain differences in initial dietary wariness, whether
421 wariness was measured as the mean time each strain took to eat the first novel food
422 item ($t = -0.77$, $P = 0.442$) or as the mean time each strain took to eat the 10th novel
423 food item ($t = -0.82$, $P = 0.412$)

424

425 *The association between dietary wariness and learning - individuals*

426 The differences in the proportion of chemically-defended prey attacked in learning
427 trial eight were not explained by individual differences in initial dietary wariness,
428 whether wariness was measured as the time each individual took to eat the first novel
429 food item ($t = -0.96$, $P = 0.408$) or the 10th novel food item ($t = -0.32$, $P = 0.746$).

430

431 *Experience and dietary wariness*

In the second dietary wariness test only three of the 20 Rhode Island Reds ate novel food, so the strain was assigned the maximum testing-time of 540s. Wariness increased significantly for all strains except ISA Warren (figure 1 white bars; 3.9 fold, 95% CI 3.3, 6.8; Wald $\chi^2_4 = 25.14$, $P < 0.001$; ISA Warren: $z = -1.29$, $P = 0.197$; Hy-Line: $z = 4.95$, $P < 0.001$; Rhode Island Red: $z = 4.44$, $P < 0.001$; and Black Rock: $z = 2.37$, $P = 0.018$). ISA Warren had a similar number of fast and slow foragers in the first and second dietary wariness test (Table 1; $\chi 2$ (1) = 0.807, $P = 0.361$), whereas all of the other strains showed an increase in the number of birds exhibiting wary behaviour after they had experienced chemical defences (Table 1; $\chi 2$ (1) = 60.667, $P < 0.0001$).

Prey handling behaviour and learning

Chicks with a higher rejection index (those that attacked but taste-rejected more chemically-defended prey during the first seven learning trials) also attacked a lower proportion of defended prey in the eighth learning trial ($t = -271$, $P = 0.008$).

Differences in learning and selection on the different prey types

Following Rowland et al (2010) we estimated the strength of selection (s) imposed by our different predators. Using the attack data from the eighth learning trial we calculated s as: $1 - ([y*nh/N]/[y*nr/N])$, where y is the number of predators, nh the number of aposematic prey attacked by the highest production strain (Hy-Line), nr the number of aposematic prey attacked by a lowest production strain (Rhode Island Red), and N is the total number of aposematic prey that could be attacked (N=160).

455 The selective difference imposed by one of each of our predators was $s = 0.14$. If we
456 multiply by 10 predators of each phenotype, selection s = 0.59.

457

458 **Discussion**

459 We predicted intra- and inter-strain differences in how chickens would react to novel
460 prey and learn about chemically defended prey (Jones, 1986), and these differences
461 would result in differential selection pressures on prey types. Our results support these
462 predictions. We hypothesised that strains of chickens selected for high production
463 traits would exhibit lower dietary wariness (consume novel prey sooner), and form
464 weaker associations between a chemical defence and warning signal (attack a higher
465 proportion of chemically-defended prey in learning trial eight), compared to strains
466 selected for lower production traits. Wariness did vary significantly between strains.
467 Chicks from the strain selected for highest annual egg productivity (Hy-Line)
468 exhibited less wariness than the strain with lowest mean annual egg productivity
469 (Rhode Island Red), but Hy-Lines were also less wary than the other strains that have
470 intermediate egg productivity (ISA Warren and Black Rock). All of the Hy-line
471 chicks were categorised as non-wary foragers in our supplementary cluster analysis,
472 whereas the other strains had a mixture of both wary and non-wary individuals.
473 Learning differed between strains: Hy-Lines attacked a higher proportion of
474 chemically defended prey in learning trial eight than the Rhode Island Reds (the strain
475 with lowest egg productivity), but did not differ to the other strains (ISA Warren and
476 Black Rock). We also predicted that strains and individuals within a strain with higher
477 initial dietary wariness would attack a lower proportion of chemically defended prey
478 in the final learning trial. Contrary to our hypotheses, the differences in strain and
479 individual learning were not explained by differences in initial dietary wariness. Our

480 data supported our prediction that experience of chemically-defended prey would
481 increase dietary wariness towards a new novel food in all strains.

482 Our results support theoretical models that predict variation in aposematic
483 signals due to differences between predators in learning and wariness (Endler, 1988;
484 Endler & Mappes, 2004; Kikuchi & Sherratt, 2015; Sherratt, 2002; Sherratt, 2011). If
485 aposematic prey were subject to attack by communities of predators that behave like
486 our Hy-Line strain, that continue to attack higher numbers of aposematic prey even
487 after learning, they might be selected to reduce their conspicuousness (this is
488 predicted in Endler & Mappes, 2004, also see results in Lindstedt et al., 2011 and
489 Valkonen et al., 2012). On the other hand, the selective pressure imposed on
490 aposematic signals by predators that attack a lower proportion of chemically-defended
491 prey than Hy-Lines, like our Rhode Island Reds, would lead to increased
492 conspicuousness (Endler & Mappes, 2004). In nature, the proportion of predators with
493 different learning strategies will likely vary from place to place and from year to year.
494 To understand the role of predator wariness and learning on aposematic signals in
495 natural systems, predator behaviour in the field should be investigated directly (this
496 point has also been made by Aubier & Sherratt 2015).

497 These varied learning strategies may be explained by differences between
498 batches within a strain rather than strain differences (note we only tested one batch
499 per strain). We think this is unlikely because, in previous research conducted by us
500 there has been no interaction between treatment and batch (Rowland et al., 2010, and
501 Rowland 2016), and the data fit our prediction and the results of other researchers
502 (Agnvall et al. 2015; Lindqvist et al. 2002), that neophobia and information
503 acquisition is reduced in the strains selected for highest production traits. Therefore,
504 we propose that the different learning strategies we have recorded are more likely due

505 to the different selection regimes our model predators have undergone, and the
506 associated differential learning costs they incur during foraging (Kikuchi and Sherratt,
507 2015).

508 Learning is affected by both extrinsic (e.g., environmental variables and prey
509 frequency: Chatelain, Halpin, & Rowe, 2013; Skelhorn & Rowe, 2007a) and intrinsic
510 factors (e.g., current physiological state: Barnett et al., 2007), that lead to trade offs
511 between the energy invested in the learning process, and the risks associated with
512 sampling potentially toxic prey (see Skelhorn et al., 2016 for a comprehensive
513 review). Hy-lines attacked a higher proportion of chemically defended prey in the
514 final learning trial compared to Rhode Island Reds. The differences in learning may
515 be due to the different energy requirements of these strains (Schütz & Jensen 2001).
516 Agnvall et al. (2015) found that metabolic differences exist between strains of
517 chickens bred for high and low fear responses, which are traits correlated with
518 domestic and commercial strains, respectively. Energetic state is known to result in
519 trade-offs in how chickens acquire information about food sources (Lindqvist et al.
520 2002; Schütz & Jensen 2001), and energy requirements have also been shown to
521 affect the foraging decisions of European starlings (*Sturnus vulgaris;* Barnett et al.,
522 2007). Starlings increase their attack rates on chemically defended insect larvae when
523 their body masses and fat stores are experimentally reduced (Barnett et al., 2007).
524 Although we attempted to keep physiological state similar across our strains (by
525 controlling the time they underwent food restriction), we did not measure metabolic
526 rates in the four strains we studied, or the effect of food restriction on their state.
527 Therefore, we think that baseline metabolic differences are a plausible explanation of
528 varied strength of learning we observed, but this remains to be tested.

A predator's ability—or how motivated it is to learn about particular prey types—may be related to its initial reaction to that prey (Schuler & Roper, 1992). When differences in predator wariness are combined with varied levels of predator learning in theoretical models, it is predicted to result in different levels of prey conspicuousness (e.g., stable equilibria of conspicous and cryptic prey in Lee et al., 2010). There is some support for the idea that wariness and learning may be connected from a study by Exnerová et al (2010), which found that fast exploring birds that were quicker to attack novel prey (less wary of novel prey) attacked more aposematic prey during learning than slow exploring birds that showed longer latencies to attack novel prey (more wary). We did not find support for the idea that a naïve predator's wariness is related to avoidance learning at the group or individual level. But we did find that dietary wariness increased in three out of four of the strains following learning to avoid chemically defended prey. Our result is in line with empirical research showing that wariness can increase after experience of defended prey (Exnerová et al., 2015; Marples et al., 2007; Schlenoff, 1984), and is predicted by an exploration-exploitation trade-off model by Sherratt (2011).

It is not clear why wariness did not change after experience with chemical defences among the ISA Warren chicks as it did among the other three strains, and has been found in other research (e.g., Marples et al., 2007). ISA Warrens did not learn differently to Hy-Line or Black Rocks (strains that did become more wary after experience), so we contend that this consistent wariness is unlikely to be due to differences in predator experience. It could be due to the specific batch of this strain we used, or could represent a real biological difference to the other three strains. Our result shows that predator species differ not only in their initial wariness, but also in how their wariness is modified by experience with different types of prey (see also

554 Adamová-Ježová et al., 2016). When a novel or uncommon aposematic prey
555 encounters an avian predator, its chance of survival will depend on that predator's
556 experience of other prey (Sherratt, 2011). Our results also emphasize the importance
557 of reporting the specific strain of chicks used in experiments on learning and
558 neophobia.

559 The methods for testing dietary wariness, and the criterion for what constitutes
560 a wary or non-wary forager vary between experiments. We found that measuring the
561 time to eat the first or tenth novel food item resulted in equivalent conclusions. In
562 addition to analyzing differences in the latency to consume novel food, we also
563 employed a cluster analysis technique (see supplementary information) to identify
564 individuals as either wary or non-wary forager. To our knowledge this is the first time
565 cluster analyses have been used to distinguish between the different foraging
566 phenotypes. This may be a useful method for future research on dietary wariness. We
567 also found that the colour of the chemically defended prey did not influence how the
568 chicks learned about those prey, but we think it is still wise to evenly divide birds in
569 each strain among colour groups as we did. One limitation of our study is that we did
570 not vary the conspicuousness of our aposematic prey. If we had presented high and a
571 low conspicuous defended prey, we could have tested if predators that form weaker
572 associations between a chemical defence and warning signal (like our Hyline strain)
573 cause higher mortality on prey with high conspicuousness, and lower mortality on
574 prey that are less conspicuous. This could show if predators that form weaker
575 associations between a chemical defence and warning signal would select for reduced
576 conspicuousness in prey. This would be a worthwhile follow-up study.

577

578 **Conclusion**

A considerable amount of the empirical research into wariness, as well as the evolutionary dynamics of warning signal evolution has used domestic chicks as model predators (Marples et al., 2007; Roper & Redston, 1987; Roper & Wistow, 1986; Rowe & Skelhorn, 2005; Skelhorn & Rowe, 2006b). Our study reveals how dependent the results of those experiments may be on the strain used.

When a novel or uncommon aposematic prey encounters an avian predator, its chance of survival will depend on that predator's experience of other prey and its motivation or capacity to learn about the prey's defences (Halpin et al., 2012; Exnerová et al., 2015). The evolution of prey defences will be affected by the community structure of naïve and experienced predators (Endler & Mappes, 2004; Nokelainen, et al., 2012).

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Appendix

Cluster analysis

We performed a cluster analysis on the data for the time to eat novel food in the first wariness assay on day two and from day seven (pre- and post-exposure to chemical defences). This allows us to look for changes in cluster position of individuals, and therefore any changes to the foraging strategy pre- and post-exposure to chemical defences.

We used the k- medoids method (Zhao 2013), which allows for smaller sample sizes, and data sets containing outliers. Unlike the k-means method (Hartigan & Wong 1979), k-medoids does not require the number of clusters to be specified prior to applying the analysis. The k-medoids method determines the most likely number of clusters. This analysis was performed in R (R Core Development Team 2013) using the 'fpc' package (Hennig 2010).

The k-medoids analysis identified two clusters within the data, which contained 95 and 20 birds respectively in the first wariness test (fig. S2 below), and 71 and 44 birds respectively in the second wariness test (fig. S3 below). The silhouette plots show that these clusters were a good fit to the data (with 1.0 being a perfect fit)

840 Table 1 The number of individuals in each strain identified as fast or slow foragers

Breed	Fast in test 1	Slow in test 1	Fast in test 2	Slow in test 2
Hyline	36	0	23	13
ISA Warren	17	7	19	5
Black Rock	30	5	26	9
Rhode Island	12	8	3	17

841 Identification of forager type was achieved by k-medoids cluster analysis (see
842 supplementary information for methods). The table shows forager type prior to
843 experiencing chemical defences (Fast1 and Slow1), and after experiencing chemical
844 defences (Fast2 and Slow2).

845

846 Figure 1. The geometric mean (GM) time in seconds to eat the first novel food item in
847 the first wariness test (grey bar) and second test after experience of chemically-
848 defended food (white bars) by each strain. Because the majority of Rhode Island Reds
849 did not consume any novel food in the second test the maximum testing-time of 540s
850 was recorded. The strains are ordered left to right from highest productivity to lowest
851 productivity.

852 Figure 2. The proportion of chemically-defended crumbs attacked per trial for each of
853 the eight learning trials. Separate lines represent each strain: black dashed line, ISA
854 Warren; black solid line, Hy-Line; grey dash line, Black Rock; and grey solid line,
855 Rhode Island Red.

856 Figure A1. The geometric mean (GM) time in seconds to eat the first (grey bar) and
857 10^{th} (white bard) novel food item in the first wariness test by each strain.

858 Figure A2. Cluster analysis results for wariness test one. On the left is a 'clusplot'
859 showing the two clusters and the distance between the clusters. On the right, the

860 silhouette plot, indicating the cluster size (n) and the associated Si (silhouette
861 information), values close to 1 indicate a perfect fit.

862 Figure A3 Cluster analysis results for wariness test two. On the left is a ‘clusplot’
863 showing the two clusters and the distance between the clusters. On the right, the
864 silhouette plot, indicating the cluster size (n) and the associated Si (silhouette
865 information), values close to 1 indicate a perfect fit.

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Author contributions

HMR conceived the study and designed experiments. GDR provided comments on the experimental design. HMR performed the experiments. HMR and AJF curated the data. HMR and AJF analysed the data. HMR wrote the manuscript. AJF and GDR provided comments on data analysis and the manuscript. All authors approved the final version of the manuscript.

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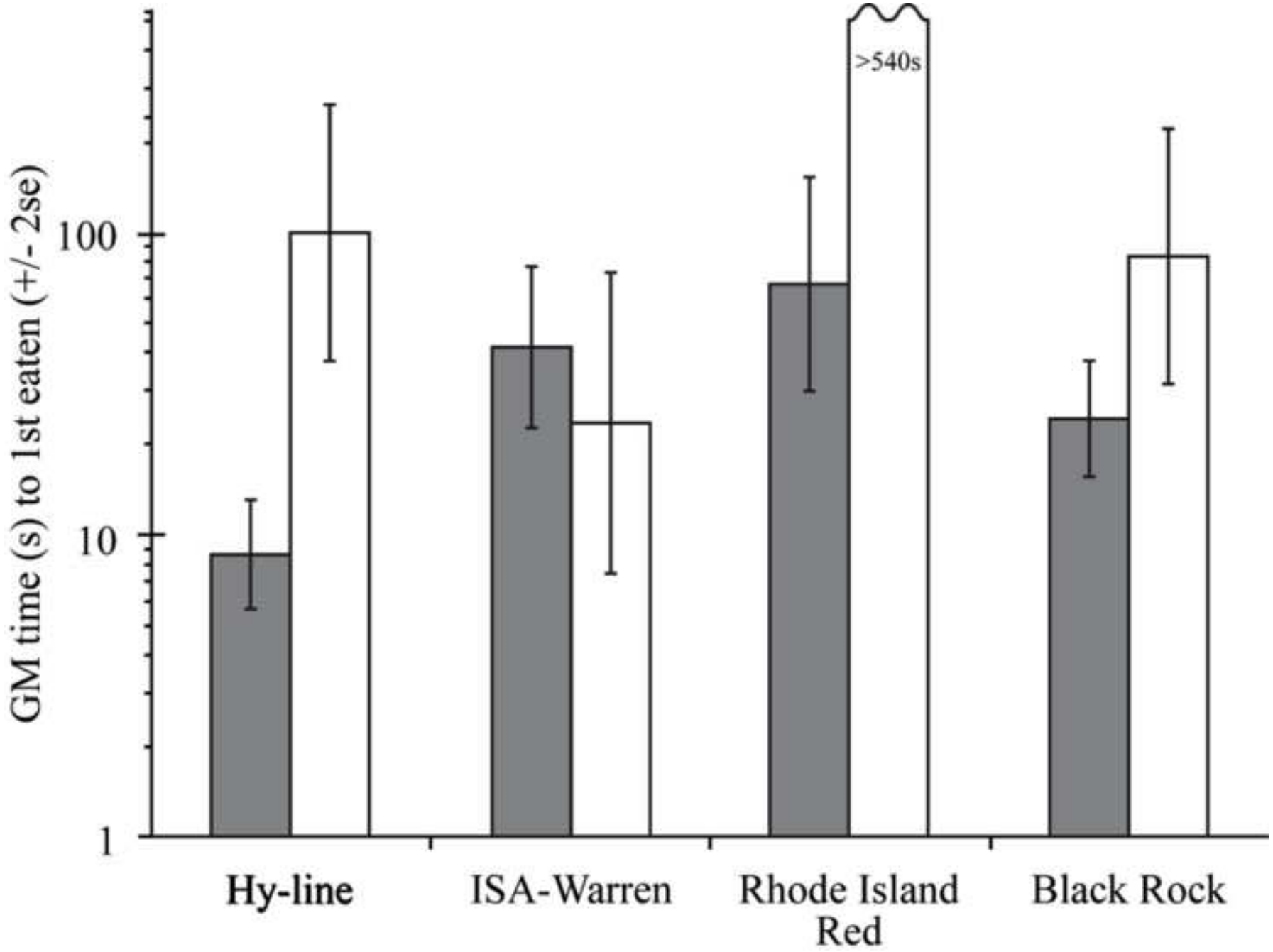

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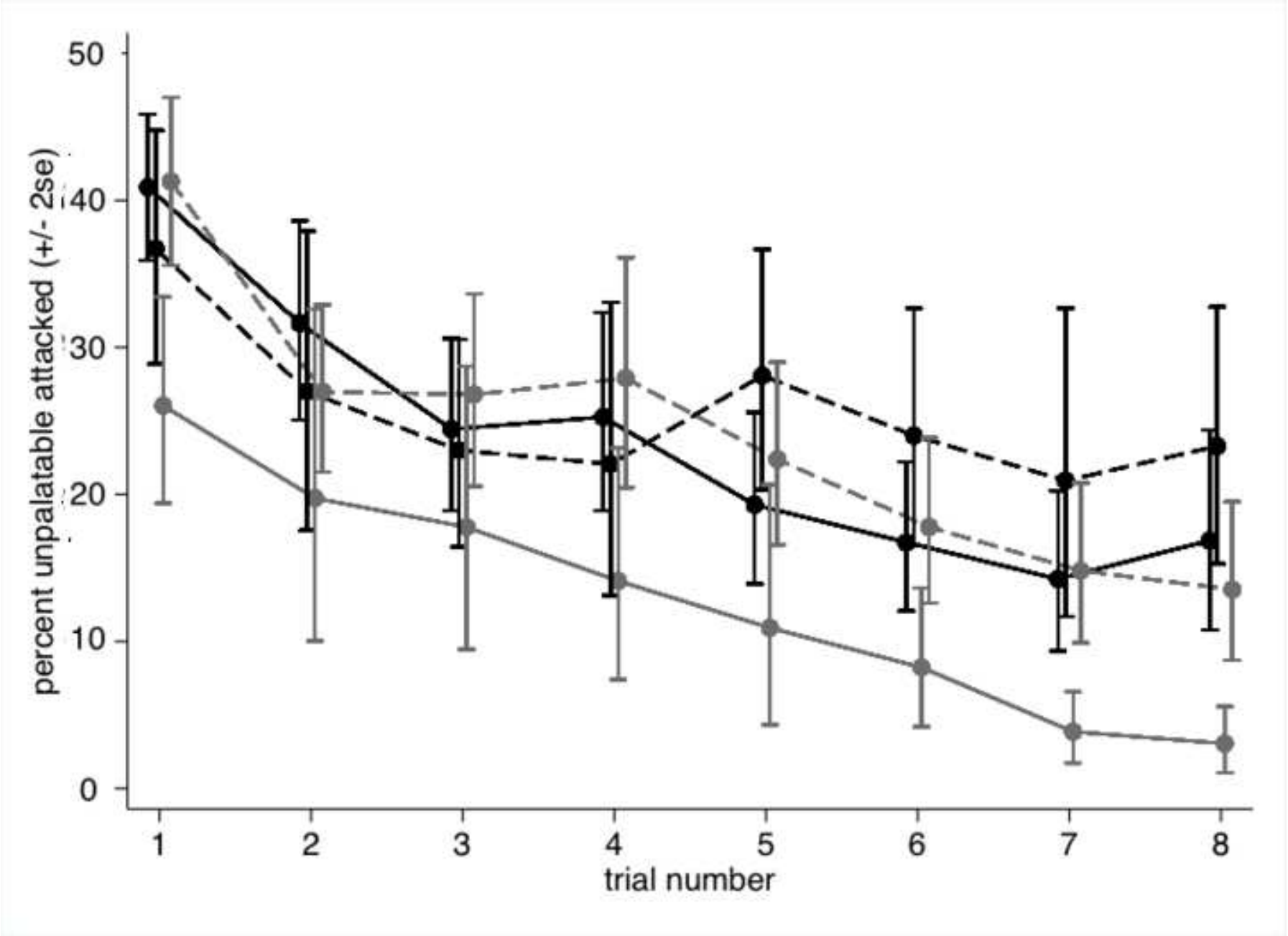

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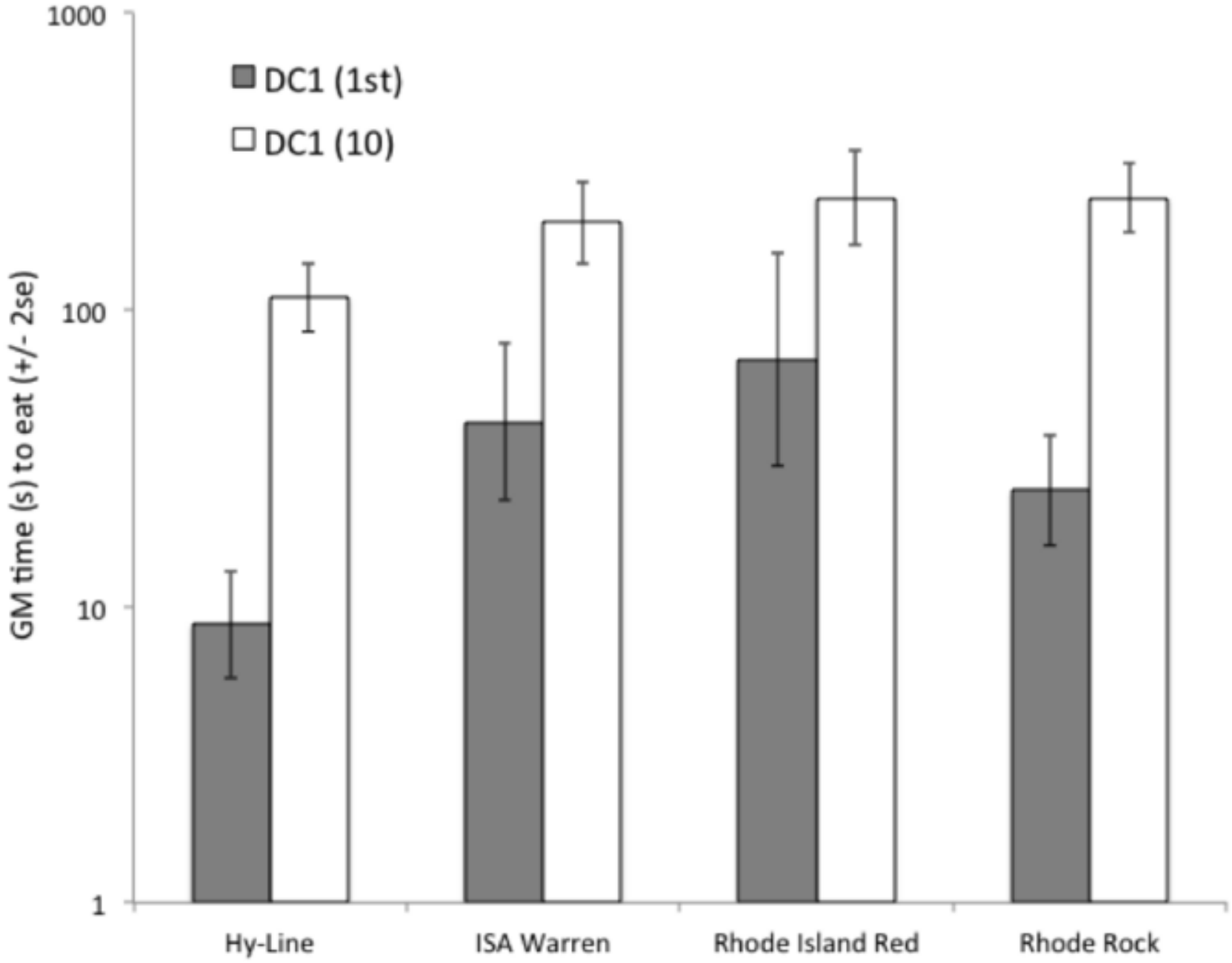

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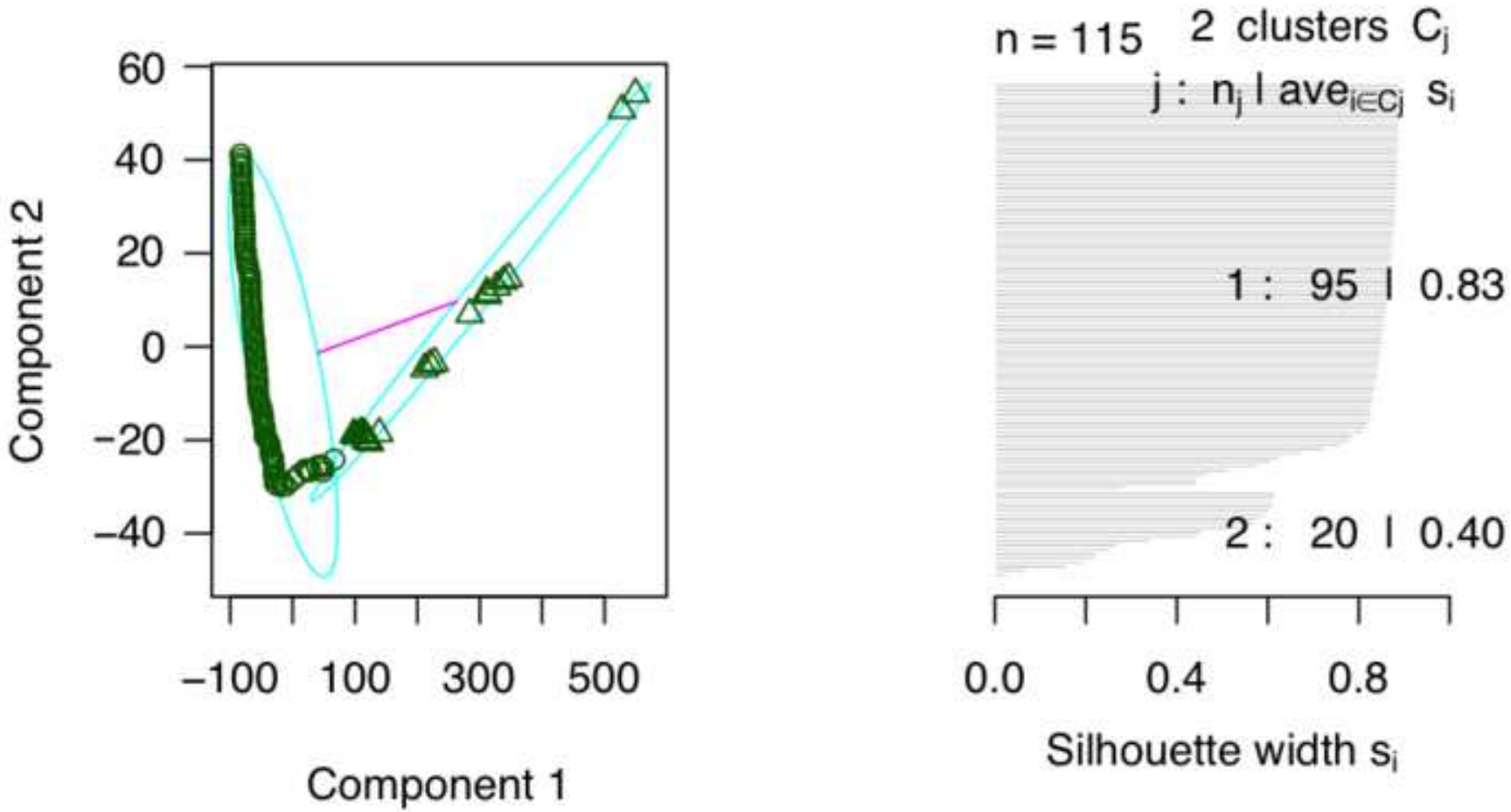

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