An ELISA for quantifying quail IgY and characterizing maternal IgY transfer to egg yolk in several quail strains

Atsushi Murai[1*], Misako Kakiuchi[1], Takahito Hamano[1], Misato Kobayashi[1], Masaoki Tsudzuki[2,3], Mikiharu Nakano[4], Yoichi Matsuda[4], and Fumihiko Horio[1]

[1]Laboratory of Animal Nutrition, Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan

[2]Laboratory of Animal Breeding and Genetics, Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Japan

[3]Japanese Avian Bioresource Project Research Center, Hiroshima University, Higashi-Hiroshima, Japan

[4]Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan

Running Head: Maternal IgY Transfer in Quail

***Correspondence:** Atsushi Murai, Laboratory of Animal Nutrition, Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan. Tel.: +81-52-789-4291; fax: +81-52-789-4077.
E-mail: atsushi@agr.nagoya-u.ac.jp

ABSTRACT

In avian species, maternal blood immunoglobulin Y (IgY) is transferred to the egg yolks of maturing oocytes, but the mechanism underlying this transfer is unknown. To gain insight into the mechanism of maternal IgY transfer in quail, we established an enzyme-linked immunosorbent assay (ELISA) for the quantitation of quail IgY. We characterized strain differences in blood and egg yolk IgY concentrations and exogenously injected IgY-Fc uptakes into egg yolks. A specific rabbit polyclonal antibody to quail IgY was raised for the ELISA. Blood and egg yolk IgY concentrations were determined in six quail strains (one inbred strain, L; four closed population strains, AWE, DB, PS, WE; one commercial strain, Commercial). The birds were also injected with digoxigenin-labeled quail IgY-Fc, and its uptakes into laid eggs were compared. The strain difference in blood and egg yolk IgY concentrations was at most 2.5-fold, between PS and AWE. The rank order of IgY concentrations was AWE, Commercial, DB, L $\geq$ WE $\geq$ PS. A significant positive correlation (|R|=0.786) between individual blood IgY and egg yolk IgY and the concentrated egg yolk IgY (1.5–2-fold) against blood IgY was observed. Interestingly, there was a significant inverse correlation (|R|=0.452) between injected IgY-Fc uptakes and the blood IgY concentration, implying competition of the injected IgY-Fc and blood IgY in the process of IgY uptake into egg yolks. In conclusion, we successfully determined blood and egg yolk IgY concentrations in various quail strains by a quail IgY-specific ELISA. The concentrated egg yolk IgY against the blood IgY and the inverse relationship of exogenous IgY-Fc uptake against the blood IgY support the existence of a selective IgY transport mechanism in avian maturing oocytes.

Keywords: Blood IgY, Egg yolk, ELISA, Maternal IgY transfer, Strain difference, Quail

Footnote

Abbreviations used: DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; PBS, phosphate-buffered saline.

1. Introduction

Avian egg yolks contains massive amounts of immunoglobulin Y (IgY), the functional equivalent of mammalian immunoglobulin G (IgG), and IgY plays a central role in the protection of the newly hatched chick against infectious disease (Kowalczyk et al., 1985). The process of avian maternal IgY transfer consists of two steps: the first step is the transfer from maternal circulating blood to the egg yolks of maturing oocytes, and the second step is the transfer from the egg yolks to the embryonic circulation through the yolk sac membrane (Linden and Roth, 1978; Tressler and Roth, 1987). The second step relies on an IgY-Fc receptor called FcRY (West et al., 2004). In the first step, however, a receptor distinct from FcRY is likely to contribute to the maternal IgY transport (Schade and Chacana, 2007; Ward, 2004). In our previous study, an Fc portion of IgY was observed to be essential for effective IgY transport into egg yolks (Kitaguchi et al., 2008b). A study using recombinant quail IgY-Fc and chicken IgY-Fc showed that a single substitution of the Tyr^{363} residue located on the Fc domain to an Ala^{363} residue seriously damages the IgY transport into egg yolks. In addition, a lack of an *N*-glycosylated carbohydrate chain at Asn^{407} on the Fc domain lowers the IgY transport into egg yolks (Murai et al., 2013; Takimoto et al., 2013). These results strongly support the existence of a specific IgY-Fc receptor mediating the uptake of blood IgY into egg yolks. However, the relevant receptor involved in IgY transport is still unidentified.

Quails are a useful experimental model for studying the mechanisms underlying how IgY is incorporated into egg yolks because of their small body size and excellent egg productivity. In our previous research, we investigated the IgY structure required for effective transport into egg yolks by performing a blood injection study of digoxigenin (DIG)-labeled IgY or its Fc fragment in Japanese quail (*Coturnix japonica*)

(Bae et al., 2010; Bae et al., 2009; Kitaguchi et al., 2008b). However, information about the endogenous blood IgY and egg yolk IgY concentrations in quail are very limited (Losonczy et al., 1999; Okuliarova et al., 2014), although these concentrations are critical parameters for overviewing the in vivo kinetics and oocyte uptake of blood IgY.

One of the reasons for the limited information about endogenous IgY concentrations in quail is that a specific antibody against chicken IgY is not always available for the detection of quail IgY by immunological measurements such as enzyme-linked immunosorbent assays (ELISAs) (personal unpubl. data), probably due to the low sequence homology (60%) of the IgY-Fc domain between quail and chicken (Choi et al., 2010). Monoclonal antibody against quail IgY was developed by Kassim et al. (2013), but blood and egg yolk IgY concentrations in quail has not been measured. It is well known that in the chicken, the endogenous blood and yolk IgY concentrations vary for multiple reasons including daily fluctuation (Carlander et al., 2001; He et al., 2014) and genetic lines or breeds (Gross and Siegel, 1990; Hamal et al., 2006; Kitaguchi et al., 2008a; Schade et al., 2005). Thus, the establishment of a quantitative analysis method for quail IgY and a method for determining the endogenous IgY concentrations in the blood and egg yolks of various quail strains would accelerate quail-based studies, elucidating the mechanism of maternal IgY transfer.

In the present study, we developed a specific polyclonal antibody against quail IgY and established a quail IgY-specific ELISA. In addition, to characterize strain differences in endogenous IgY concentrations and to gain insight into the mechanisms of maternal IgY transfer in quail, we determined the blood and egg yolk IgY concentrations and exogenously injected IgY-Fc uptakes into egg yolks in six quail strains (one inbred, four closed colony, and one commercial strain).

2. Materials and methods

2.1. *Animals*

Commercial female Japanese quail were purchased from a local hatchery (Cyubu-kagaku-shizai, Nagoya, Japan). Female quail of one inbred strain (L, Low antibody production to Newcastle disease virus) and four closed colony strains (AWE, Albino White Egg shell; DB, Dominant Black color; PS, PanSy plumage color; WE, White Egg shell) were supplied by the National BioResource Project – Chicken and Quail, Nagoya University (Nagoya, Japan). All birds used here were unvaccinated. The birds were maintained individually with free access to water and a commercial diet (Mash-Neo®; Toyohashi Feed Mills, Toyohashi, Japan). The photoperiod was set at 16L:8D during the experiment. The room temperature was controlled at 23±2°C. Egg production was recorded daily, and continuously laying birds at 11–45 weeks of age were used for the animal experiments. The animal care was in full compliance with the guidelines of the Nagoya University Policy on Animal Care and Use.

2.2. Production of recombinant quail IgY-Fc

Recombinant quail IgY-Fc (residues 231–568 of the Fc domain; Choi et al., 2010) with a C-terminal 6×his tag was produced as described (Takimoto et al., 2013). We used the purified IgY-Fc for the production of the polyclonal antibody and the injection study for the measurement of the IgY-Fc uptake into egg yolks. For the injection study, the IgY-Fc was labeled with DIG (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s recommendations.

2.3. Antibody production

To obtain a highly specific antibody against quail IgY, we used the purified quail IgY-Fc as an antigen. The IgY-Fc at 200 μg in 1 mL of phosphate-buffered saline (PBS) was emulsified with 1.5 mL of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO) for the first and second immunizations. The prepared immunogen was subcutaneously injected into a 4-month-old female New Zealand White rabbit at 2-week intervals. For the third and fourth immunizations, the IgY-Fc was mixed with Freund's incomplete adjuvant, and then the immunogen was administered to the rabbit in the same way. Four days after the fourth immunization, the blood serum was collected and an increased antibody titer was confirmed by an ELISA. After that, 20–50 mL of blood was collected from the rabbit several times, and the pooled serum was mixed with ammonium sulfate for the precipitation of antibodies. The pellets of antibodies were resuspended in 2 M Tris-buffered saline (pH 7.4).

For the isolation of the quail IgY-specific antibody, we applied the resuspended solution to affinity chromatography consisting of a HiTrap NHS-activated HP column (GE Healthcare, Waukesha, WI) conjugated with native quail IgY purified from quail egg yolks (Bae et al., 2009). Part of the isolated antibody was labeled with horseradish peroxidase (HRP) with the use of a peroxidase labeling kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan). To check the specificity of the raised antibody, we performed a Western blotting analysis of the HRP-conjugated antibody (1:10,000) for the detection of graded levels (0.1–2 ng/lane) of native quail IgY and native chicken IgY (Rockland Immunochemicals, Gilbertsville, PA).

2.4. Collection of blood and yolk samples and preparation of yolk extract

151 Six strains of regularly laying quails were used for the measurements of blood and egg
152 yolk IgY concentrations and the injected IgY-Fc uptake into egg yolks. Blood samples
153 were collected via the wing vein twice at a 2-week interval. The blood samples were
154 centrifuged at 10,000*g* for 4 min at 4°C. The supernatant was collected and stored as the
155 serum sample. At 1 week after the first blood sampling, each quail within several hours
156 of oviposition was injected intravenously with 20 µg/100 g body weight of DIG-labeled
157 IgY-Fc. Laid eggs were collected for 2 and 3 days after the injection and stored at 4°C
158 until the analysis.

159 Yolk extract of IgY was prepared as described in Takimoto et al. (2013), which
160 is modified version of the water dilution method by Akita and Nakai (1993) and
161 Kawabe et al. (2006). Briefly, the egg white was removed from the egg yolk by passing
162 throw wire mesh sheet with 6 mm square space, and the remaining egg yolk on the sheet
163 was used for the measurement of incorporated proteins. The yolk membrane was
164 punctured, and the whole yolk was allowed to drain into a 50-ml polypropylene tube
165 and then mixed. One gram of the well-mixed yolk was transferred into a 50-ml
166 polypropylene tube. The yolk sample was diluted with five volumes of PBS and stored
167 at 4°C overnight, then centrifuged at 10,000*g* for 25 min at 4°C. The supernatant was
168 collected and filtered through Whatman No. 2 filter paper. This final solution was used
169 for the determination of the concentrations of the injected IgY-Fc and total IgY by the
170 new ELISA described below.

171

172 *2.5. Quantitation of quail IgY and DIG-labeled IgY-Fc by ELISA*

173 We used the isolated antibody against quail IgY-Fc (1 mg/mL) and its complex
174 conjugated with HRP for the measurements of the blood and yolk IgY concentrations by

175 an ELISA. Microtiter plates with 96 wells were coated with 100 µl/well of the isolated
176 antibody (1:500) in 50 mM sodium carbonate (pH 9.6) for 60 min at room temperature.
177 After the washing, the plates were filled with 200 µl/well of blocking solution
178 containing 1% (v/v) bovine serum albumin (A7284; Sigma-Aldrich, St. Louis, MO) and
179 incubated for 30 min. Subsequently, the plates were incubated with standards and
180 samples at 100 µl/well for 1 h. The standards were serially diluted within the range of
181 1–1,000 ng/ml of native quail IgY. The plate was then washed and incubated for 1 h
182 with 100 µl/well of the HRP-conjugated antibody (1:16,000). The plates were washed
183 and a color reaction was initiated by adding 100 µl *o*-phenylendiamine solution (a
184 solution of 50 mM citric acid and 50 mM Na_2HPO_4, pH 5.0 containing an
185 *o*-phenylendiamine tablet [Sigma-Aldrich, St. Louis, MO] with 0.01 % [v/v] H_2O_2) for
186 5 min.

187 The reaction mixture was terminated by adding 100 µl of 3 M H_2SO_4, and
188 absorbance was measured at the wavelength of 490 nm with a microtiter plate reader.
189 The concentrations of DIG-labeled quail IgY-Fc in the egg yolk extracts were measured
190 by an ELISA that detects conjugated DIG (Bae et al., 2009).

191

192 *2.6. Data analysis*

193 We performed a one-way ANOVA to analyze the results. The mean values of body
194 weight, blood and egg yolk IgY concentrations, and uptake of quail IgY-Fc were
195 compared by Tukey-Kramer's test. All error bars were expressed as the standard error
196 of the mean (SEM), and differences between means were considered significant at
197 $P<0.05$. Pearson correlation coefficients in the measured parameters (blood IgY vs. egg
198 yolk IgY; IgY-Fc uptake vs. blood IgY; IgY-Fc uptake vs. yolk IgY) were calculated.

3. Results

3.1. Specificity of anti-quail IgY antibody and the establishment of the ELISA

With the use of the anti-quail IgY-Fc antibody raised in the present study, the native quail IgY and native chicken IgY were detected by our Western blotting analysis (Fig. 1A). The quail IgY migrated as a clear single band with the apparent molecular mass of 180 kDa under the non-reducing condition and 70 kDa under the reducing condition. Graded amounts of quail IgY were detected as different dense bands in a dose-dependent manner.

In contrast, the detection of chicken IgY was approx. 20-fold lower than that of quail IgY. Serial dilutions of quail IgY ranging from 1 to 1,000 ng per ml produced dose-dependent antibody titration curves in the ELISA (Fig. 1B). The half-maximal absorbance of the quail IgY standard curve was reached at approx. 70 ng/ml. These results showed that the antibody raised against quail IgY-Fc can specifically detect quail IgY, and that the ELISA established herein can be used for the quantitation of quail IgY.

3.2. Strain differences in blood and egg yolk IgY concentrations

The quail strains' body weights and collected egg weights are shown in Table 1. The body weights of the L strain were the highest among the six strains, and the AWE body weights were the lowest. The difference in body weight between the L and AWE strains was only 10% of their weights. There were no significant differences in egg weight or yolk weight among any of the strains.

We used the new ELISA to measure the total IgY concentrations in blood and egg yolks (Fig. 2A,B). The AWE quails had the highest blood and egg yolk IgY

concentrations, and the PS strain had the lowest IgY concentrations. The WE quails had medium-level blood IgY and egg yolk IgY concentrations. The rank order of IgY concentrations in all strains was AWE, Commercial, DB, L ≥ WE ≥ PS. A significant positive correlation between the individual blood IgY and egg yolk IgY was observed (|R|=0.786, P<0.0001, n=43; Fig. 2C). The concentration factor of egg yolk IgY against blood IgY was approx. 1.5–2.0, and there was no remarkable difference in this factor among the strains.

3.3. Injected IgY-Fc uptake into egg yolks

We first examined the uptake pattern of injected IgY-Fc into egg yolks of sequentially laid eggs in the L and AWE strains. Following the injection of IgY-Fc, its uptake into egg yolks was undetectable at 1 day after injection, but peaked at 2 and 3 days after injection in both strains (Fig. 3). We observed that the combined uptakes of injected IgY-Fc at 2 and 3 days occupied two-thirds of their total uptakes. We therefore used the averaged uptakes at 2 and 3 days after injection for the comparison of the IgY-Fc uptakes among the six quail strains.

The uptakes of injected IgY-Fc into egg yolks did not differ significantly among the strains, although the L (highest) and DB (lowest) strains showed an approx. 45% difference in their uptakes (Fig. 4A). Interestingly, the rank order of the injected IgY-Fc uptakes is opposite that of the endogenous blood and egg yolk IgY concentrations (Fig. 4A vs. Fig. 2AB), and we therefore calculated the correlation between the injected IgY-Fc uptakes and the endogenous IgY concentrations. There was a significant inverse correlation between the IgY-Fc uptakes and blood IgY concentrations (|R|=0.452, P<0.0016, n=46; Fig. 4B). A similar characteristic inverse correlation was also observed

247 between the IgY-Fc uptakes and the egg yolk IgY concentrations (|R|=0.483, P<0.001,
248 n=43; Fig. 4C). Taken these results together, it is apparent that although no significant
249 strain difference in injected IgY-Fc uptakes was observed, there was a negative
250 correlation between the injected IgY-Fc uptakes and the endogenous IgY concentrations.
251
252 **4. Discussion**
253 In this study, we successfully obtained a specific polyclonal antibody against quail
254 IgY-Fc. Using this antibody, we observed that the PS and WE quail strains had lower
255 blood and egg yolk IgY concentrations than those of the Commercial, AWE, DB and L
256 strains. The strain difference in the blood and egg yolk IgY concentrations was at most
257 2.5-fold between the PS and AWE strains. Predictably, the egg yolk IgY concentrations
258 were proportional to the blood IgY concentrations, implying that strain differences in
259 the egg yolk IgY concentration are attributable to strain variations in blood IgY
260 concentrations. The present results in quail are consistent with the previous findings for
261 chickens (Hamal et al., 2006; Kitaguchi et al., 2008a). The blood IgY concentration is
262 thus a critical factor in the egg yolk IgY concentration in both quail and chickens.
263 To the best of our knowledge, there have been no reports of quail or chicken
264 strains producing eggs with an IgY concentration that is not proportional to the blood
265 IgY concentration, suggesting that the maternal IgY transfer system is a fundamental
266 and basic physiological phenomenon to ensure survival of the offspring. In fact,
267 (Grindstaff, 2008) reported that growth-suppressive effects of antigen exposure during
268 the development of quail offspring are ameliorated by the presence of maternal
269 antibodies, but in the absence of specific maternal antibodies, the offspring must
270 consume their ability and energy source to protect the antigen. On the other hand, if a

quail or chicken strain exists that has a distinctive egg yolk IgY concentration that is not proportional to its blood IgY concentration, the strain would be a useful experimental model for studying the mechanisms underlying maternal IgY transfer in avian species.

The concentration factor of egg yolk IgY against blood IgY provides a clue to the mechanism by which blood IgY is selectively transported into ovarian oocytes. In our previous study, the concentration factor of egg yolk IgY against blood IgY was approx. 1.7 regardless of the chicken strain (Kitaguchi et al., 2008a). The same result was obtained in the present study; the concentration factor of egg yolk IgY against blood IgY was 1.5–1.7 in all quail strains. Vitellogenin, an egg yolk precursor synthesized in the liver, is incorporated into maturing oocytes by vitellogenin/VLDL receptor expressed in the oocyte plasma membrane. The concentration of egg yolk phosvitin, a component of vitellogenin, was nine-fold higher than that of blood phosvitin (Cutting and Roth, 1973). Riboflavin and its binding protein are also incorporated into maturing oocytes by the same vitellogenin/VLDL receptor, and they are concentrated six-fold during blood-to-egg yolk transfer (Mac Lachlan et al., 1994). The concentration factor of quail IgY (1.5–1.7) was considerably lower than those of vitellogenin and riboflavin binding protein. However, the richness of IgY in blood [approx. 10-fold higher against vitellogenin (Roth et al., 1976) and 10^6-fold higher against riboflavin (Naber, 1993)] should be considered in the interpretation of IgY enrichment in egg yolks.

The results of our injection study of quail IgY-Fc and its correlation analysis against endogenous IgY also provide clues about how maturing oocytes regulate IgY transfer. A remarkable strain difference in injected IgY-Fc uptakes into egg yolks was not observed in this study; interestingly, however, the IgY-Fc uptakes were inversely correlated with endogenous blood IgY when all strain data were pooled. The most

295 plausible explanation is that the injected IgY-Fc competed with the endogenous blood
296 IgY during the transfer process, which resulted in the reduced IgY-Fc uptakes into eggs.
297 The present data provide indirect evidence of the presence of a unique IgY transfer
298 system in avian oocytes, probably due to receptor-mediated endocytosis.

299 In our previous study, a single amino acid substitution of the Tyr^{363} residue
300 located on the Fc domain to an Ala^{363} residue seriously damaged the IgY-Fc transport
301 into egg yolks (Takimoto et al., 2013). In addition, the comprehensive substitution of
302 Tyr^{363} with other amino acids revealed that the residues at Tyr^{363} needs to be allocated
303 with aromatic amino acids to maintain the IgY-Fc transport ability (Murai et al., 2013).
304 In fact, CHIR-AB1, a member of the leukocyte receptor family found in chicken, did
305 not bind to an IgY mutant with a substitution of Tyr^{363} residue to Ala^{363} (Pürzel et al.,
306 2009). However, CHIR-AB1 seems to have limited relevance in the IgY transport into
307 egg yolks because of its scarce gene expression in ovarian follicles (unpubl. data). FcRY
308 is responsible for the transfer of yolk IgY to embryonic circulation. However, relevance
309 of FcRY on maternal blood IgY to the egg yolks is unlikely, because human IgG does
310 not bind to FcRY (West et al., 2004) even though human IgG is transported into egg
311 yolks. In addition, FcRY gene expression is undetectable in the ovarian inner layers
312 (Kitaguchi et al., 2010). Taken the present results and recent reports together, we
313 propose again that unidentified receptor involved in maternal IgY transfer exists in the
314 maturing oocytes in avian species.

315 In conclusion, we successfully determined the blood and egg yolk IgY
316 concentrations in various quail strains by performing our novel quail IgY-specific
317 ELISA. Our findings confirmed that strain differences in egg yolk IgY concentration are
318 caused by a strain difference in blood IgY concentrations. Both the egg yolk IgY

319 concentration against the blood IgY concentration and the inverse relationship of the
320 exogenously injected IgY-Fc uptake and the blood IgY support the existence of a
321 selective IgY transport mechanism in avian maturing oocytes. Thus, quail as well as
322 chicken are useful models to clarify the mechanisms of maternal IgY transfer in avian
323 species.
324

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329 quail strains (L, AWE, DB, PS, WE).
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Figure legends

Fig. 1. Specificity of the anti-quail IgY-Fc antibody and the standard curve of quail IgY by the ELISA. (A) Native quail IgY and chicken IgY at graded levels were detected by Western blotting under reducing and non-reducing conditions by using the anti-quail IgY-Fc specific antibody. (B) Free anti-quail IgY-Fc antibody and its complex conjugated with HRP were used for the ELISA. The standards were serially diluted within the range of 1–1,000 ng/ml of native quail IgY.

Fig. 2. The blood IgY (A) and egg yolk IgY (B) concentrations of a commercial quail strain and five quail strains. Blood and egg yolk samples were collected, and their IgY concentrations were measured by the ELISA. Vertical bars: mean±SEM. The numbers in parentheses under the names of the lines indicate the number of birds in each strain. ab; $P<0.05$. (C) The correlation between the blood IgY and egg yolk IgY concentrations. The plotted data are the same as the data of panels (A) and (B). |R|, correlation coefficient.

Fig. 3. The uptake of injected quail IgY-Fc into the egg yolks of L and AWE strains. DIG-labeled quail IgY-Fc (20 µg/100 g body weight) was injected intravenously into laying quail, and its uptakes into egg yolks were measured by the ELISA. Vertical bars: means±SEM of 3–5 eggs.

Fig. 4. The uptake of injected quail IgY-Fc into the egg yolks of the six quail strains. (A) DIG-labeled quail IgY-Fc (20 µg/100 g body weight) was injected intravenously

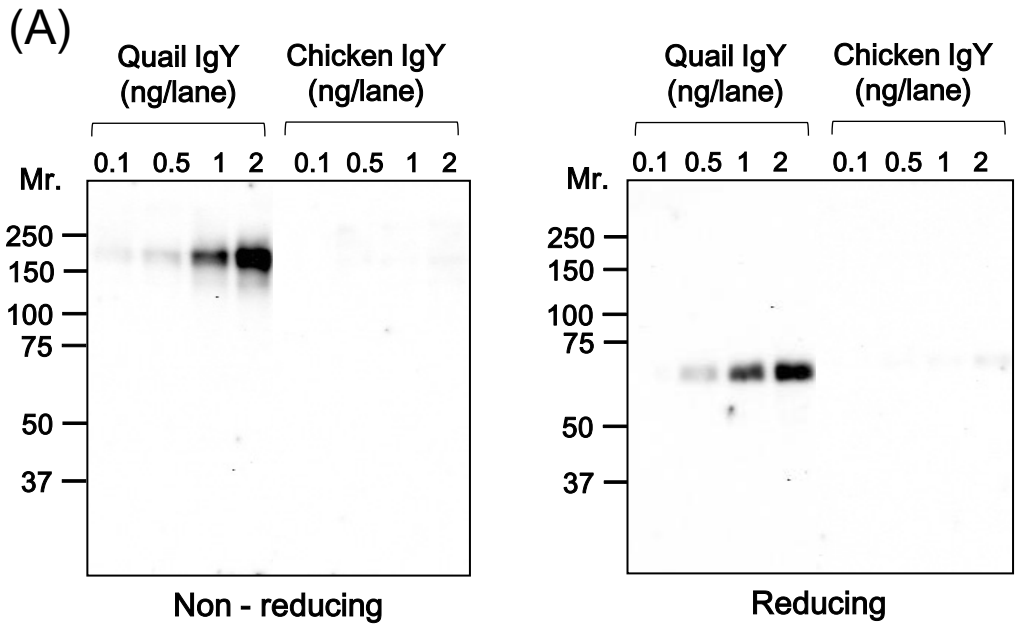
444 into laying quail, and its uptakes into egg yolks were measured by the ELISA. The
445 averaged uptakes into eggs laid 2 and 3 days after the injection were compared among
446 the strains. Vertical bars: means±SEM. The numbers in parentheses under the names of
447 the lines indicate the number of birds in each strain. (B, C) The correlations between the
448 uptake of injected quail IgY-Fc and the blood IgY concentration (B) or the egg yolk IgY
449 concentration (C). The blood IgY and egg yolk IgY concentration data are the same as
450 those shown in Figure 2. |R|, correlation coefficient.

Table 1. Body weight, egg weight and yolk weight of a commercial quail and five quail strains

	Commercial	AWE	DB	L	PS	WE
Body weight (g)	139.9±1.1^{ab}	136.2±2.3^{b}	145.1±3.2^{ab}	149.9±3.2^{a}	147.5±2.7^{ab}	144.7±3.6^{ab}
Egg weight (g)	10.1±0.34	9.92±0.30	10.6±0.37	9.32±0.34	9.71±0.34	9.50±0.27
Yolk weight (g)	2.86±0.091	2.78±0.085	3.08±0.11	2.74±0.11	2.97±0.073	2.86±0.19

Values are mean ± SEM (Commercial, n=6; AWE, n=11; DB, n=7; L, n=8; PS, n=8; WE, n=6).

^{ab}; Means having different superscripts are significantly different at $P < 0.05$.


(A)
Quail IgY (ng/lane)
Chicken IgY (ng/lane)
0.1 0.5 1 2 0.1 0.5 1 2
Mr.
250
150
100
75
50
37
Non - reducing
Quail IgY (ng/lane)
Chicken IgY (ng/lane)
0.1 0.5 1 2 0.1 0.5 1 2
Mr.
250
150
100
75
50
37
Reducing

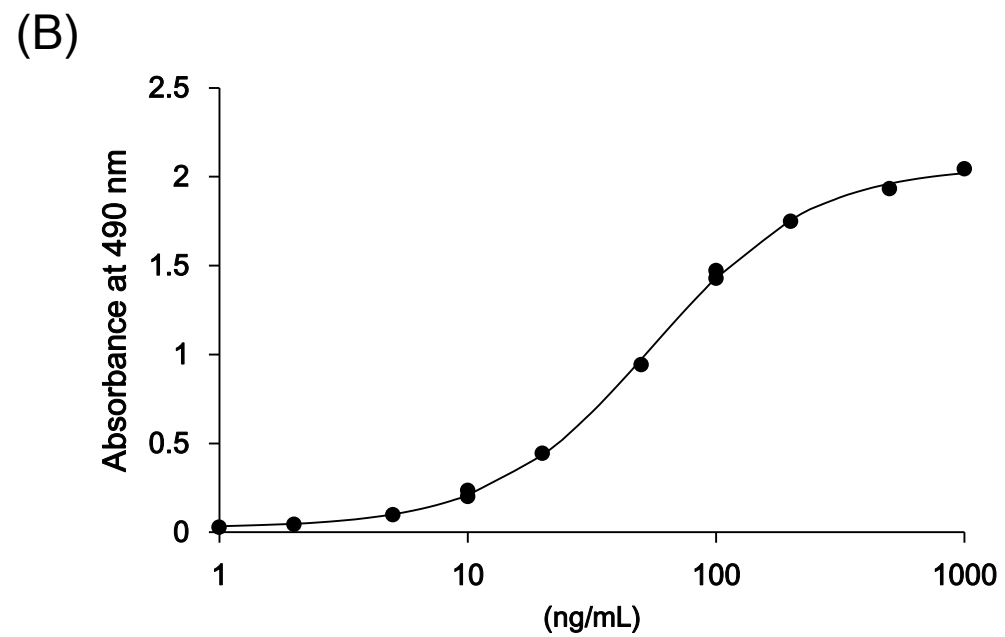

(B)
Absorbance at 490 nm
2.5
2
1.5
1
0.5
0
1
10
100
1000
(ng/mL)

Figure 1

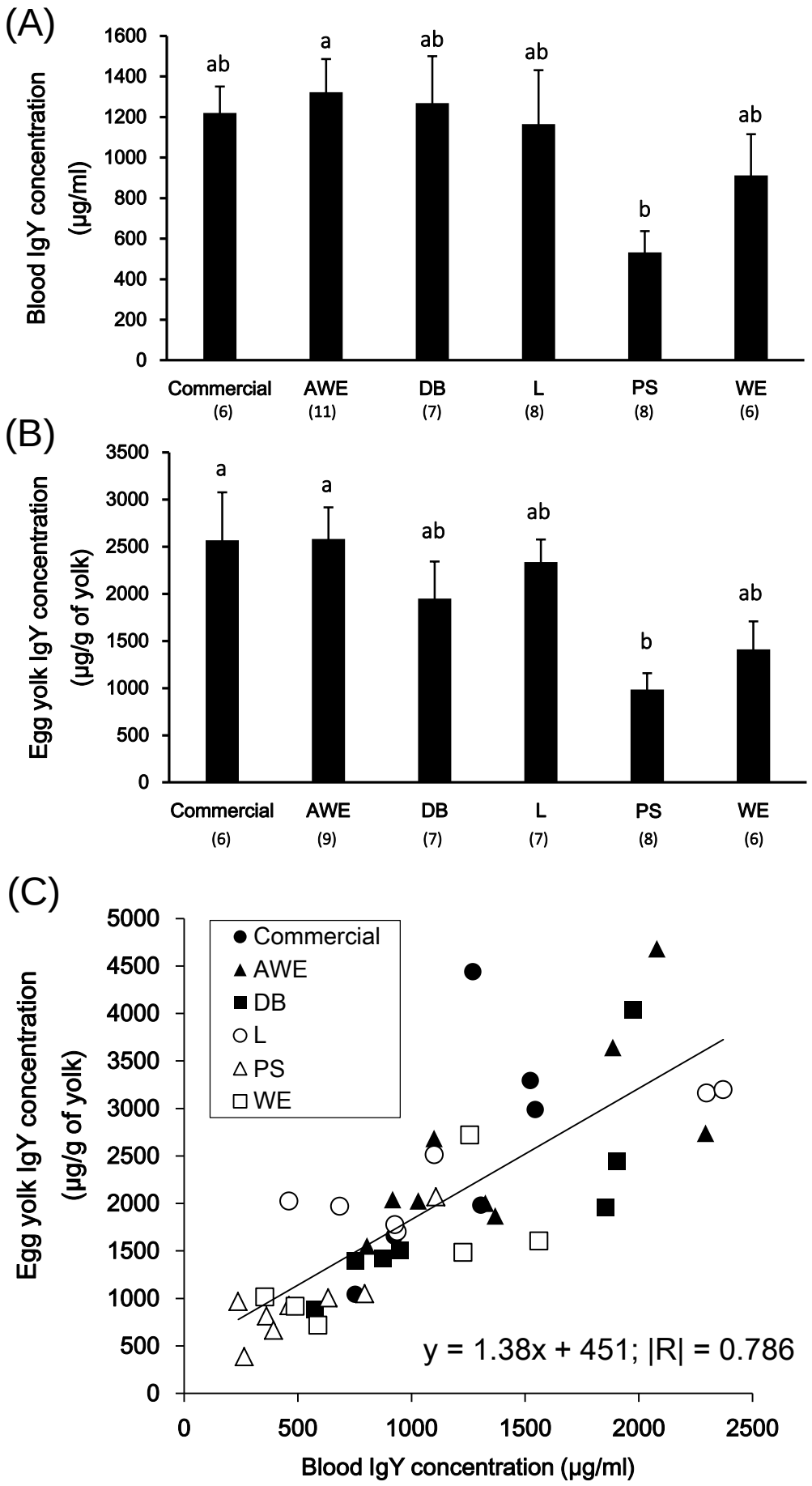
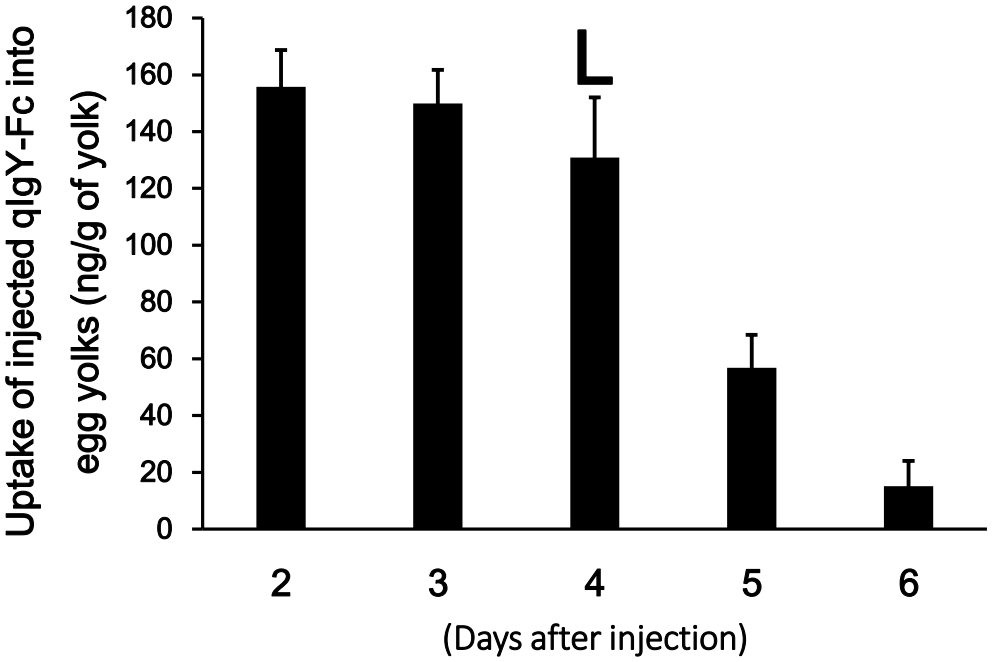

(A)
Blood IgY concentration (µg/ml)
1600
1400
1200
1000
800
600
400
200
0
ab
a
ab
ab
b
ab
Commercial (6)
AWE (11)
DB (7)
L (8)
PS (8)
WE (6)
(B)
Egg yolk IgY concentration (µg/g of yolk)
3500
3000
2500
2000
1500
1000
500
0
a
a
ab
ab
b
ab
Commercial (6)
AWE (9)
DB (7)
L (7)
PS (8)
WE (6)
(C)
Egg yolk IgY concentration (µg/g of yolk)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Commercial
AWE
DB
L
PS
WE
y = 1.38x + 451; |R| = 0.786
0
500
1000
1500
2000
2500
Blood IgY concentration (µg/ml)

Figure 2

(A)


Uptake of injected qIgY-Fc into egg yolks (ng/g of yolk)
180
160
140
120
100
80
60
40
20
0
L
2
3
4
5
6
(Days after injection)

(B)


Uptake of injected qIgY-Fc into egg yolks (ng/g of yolk)
140
120
100
80
60
40
20
0
AWE
2
3
4
5
6
(Days after injection)

Figure 3

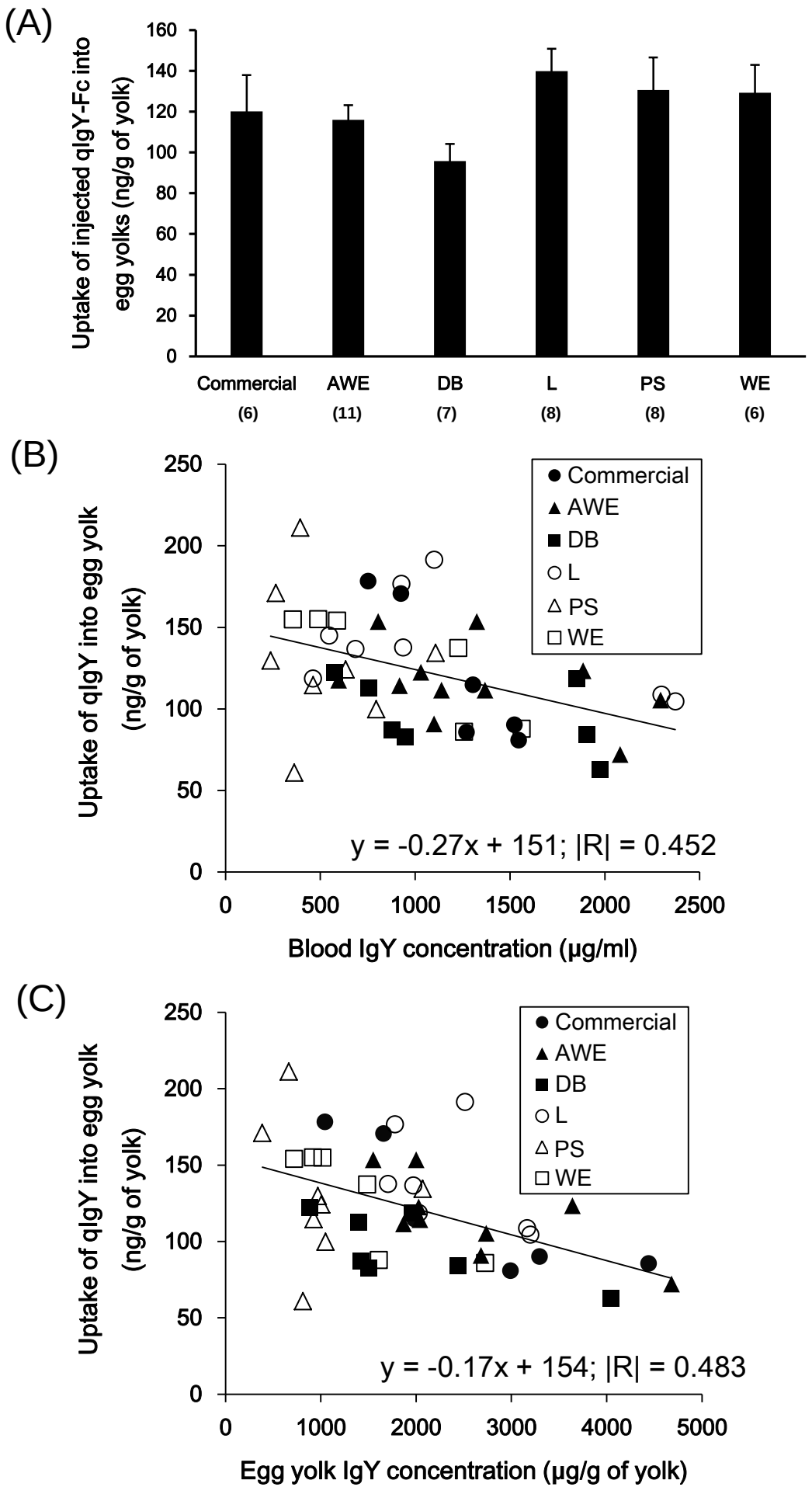

(A)
Uptake of injected qIgY-Fc into egg yolks (ng/g of yolk)
160
140
120
100
80
60
40
20
0
Commercial (6)
AWE (11)
DB (7)
L (8)
PS (8)
WE (6)
(B)
Uptake of qIgY into egg yolk (ng/g of yolk)
250
200
150
100
50
0
Commercial
AWE
DB
L
PS
WE
y = -0.27x + 151; |R| = 0.452
0
500
1000
1500
2000
2500
Blood IgY concentration (µg/ml)
(C)
Uptake of qIgY into egg yolk (ng/g of yolk)
250
200
150
100
50
0
Commercial
AWE
DB
L
PS
WE
y = -0.17x + 154; |R| = 0.483
0
1000
2000
3000
4000
5000
Egg yolk IgY concentration (µg/g of yolk)

Figure 4