

Monitoring of laying capacity, immunoglobulin Y concentration, and antibody titer development in chickens immunized with ricin and botulinum toxins over a two-year period

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ABSTRACT One of the key benefits in using chickens for immunization is the high yield of antibodies obtainable. It is known that egg production decreases over time, while animal maintenance costs remain stable. It would, however, be desirable to keep hens as long as possible to obtain maximal amounts of antibodies. To identify a suitable length of time that animals can be kept and to optimize the cost:yield ratio, we monitored the number of eggs laid, the total amount of chicken IgY, and the specific antibody titer from individually prepared eggs over a 2-yr period. The plant toxin ricin and the *Clostridium botulinum* neurotoxins type A and B were used to immunize 4 chickens. The number of eggs laid in 2 yr was approximately 600 per hen (about 80% of the maximum egg number), yielding about 20 to 40 g of total IgY per hen. A stable antibody titer of 1:100,000 to 1:1,000,000, as measured by ELISA, was obtained following up to 11 injections of 10 to 20 µg of

immobilized native toxin. Laying capacities were found to decrease, on average, from 7 eggs/wk at the point of first immunization to 2 eggs/wk after more than 2 yr. In parallel, the yield of total and specific IgY increased over time, so that the antibody recovery remained high, even after prolonged immunization times. Using purified IgY preparations, classical immunological assays such as ELISA and Western blotting were performed. Furthermore, the IgY showed neutralizing capacity when used to block the functional activity of the toxins both in vitro and in vivo. Analysis of the total IgY content over time demonstrated a complex biological oscillation (and the antigen-specific titer), with a shorter time period of around 7 d (circaseptan rhythm). In summary, we successfully immunized chickens with ricin and botulinum neurotoxins and monitored laying capacity, IgY concentration, and specific antibody titer over an extended period of 2 yr.

Key words: ricin, botulinum neurotoxin, immunoglobulin Y content, laying capacity, circaseptan rhythm

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INTRODUCTION

Immunoglobulin Y technology—the production of polyclonal antibodies in chickens and the extraction from egg yolk—is an innovative and expanding branch of biotechnology. In contrast with the more commonly used mammalian IgG antibodies, IgY antibodies do not activate mammalian complement, nor do they cross-react with Fc-receptors, mammalian rheumatoid factor, or human anti-mouse antibodies, thus eliminating the cause of false-positive results in immunological assays. Moreover, IgY can easily be sampled noninvasively from the egg yolk, compared with the stressful bleeding of animals to obtain serum. From an economical point

of view, the antibody production of a hen roughly corresponds to that of a large mammal such as a sheep or goat, whereas maintenance costs are relatively low (Larsson et al., 1993; Schade et al., 2001, 2005; Zhang, 2003). Thus, a substantial amount of antibody can be produced from just one hen (up to 40 g of total IgY per chicken per year), of which 1 to 10% can be expected to be antigen-specific (Mine and Kovacs-Nolan, 2002). This vast quantity of antibodies may pave the way for new fields of application such as immunotherapy and immunoprophylaxis. Chickens are able to respond better to mammalian antigens providing specific IgY antibody because of the phylogenetic distance between the animal classes *Mammalia* and *Aves*, as for instance the rabbit immune system.

Hens are routinely immunized for periods between a few weeks and several months, depending on the study objective, as well as on the amount of antibody needed. Large amounts of antibody are required for the pro-

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duction of therapeutic antibodies, and this could be achieved either by increasing the number of animals used or by extending the maintenance period (De Ceuninck et al., 2001; Kollberg et al., 2003; Kovacs-Nolan and Mine, 2005). The aim of the current work was to generate large amounts of antibody for immunological detection of toxins, and to identify a suitable period that animals could be kept to ensure high yields of specific antibodies at a reasonable cost. Toxins with significance in bioterrorism such as the plant toxin ricin and the botulinum neurotoxins type A and B (**BoNT/A**, **BoNT/B**) were therefore chosen for these immunization studies.

Castor beans (*Ricinus communis*) contain large amounts (up to 5%) of the highly toxic lectin ricin and a highly homologous lectin named *R. communis* agglutinin (RCA 120) of lower toxicity. In mice, the half-maximal lethal dose (**LD**₅₀) of ricin is around 5 µg/kg of BW when inhaled or after i.v. injection (Bradberry et al., 2003). Ricin consists of a 30- to 32-kDa A-chain and a 33-kDa B-chain, whereas agglutinin occurs as a tetramer of 2 A- and 2 B-chains of similar size to those of ricin (Olsnes et al., 1974). On the molecular level, the B-chain binds to sugar residues on the cell surface and facilitates the uptake and release of the A-chain into the cytoplasm. In the cytoplasm the A-chain, an RNA *N*-glycosidase, inactivates the ribosome by removing an adenine from 28S ribosomal RNA (Lord et al., 2003). The abundance of the castor plant, the ease with which crude ricin may be extracted from the seeds, and the toxicity make ricin a relevant candidate substance for bioterrorism. Ricin is classified as a category B agent by the Centers for Disease Control and Prevention and it falls under the Organisation for the Prohibition of Chemical Weapons regulation of chemical weapons (Audi et al., 2005).

The gram-positive bacterium *Clostridium botulinum* produces 7 types of neurotoxins (A to G), known as the most toxic substances on earth, with an LD₅₀ of approximately 1 ng/kg of BW after injection or inhalation (Arnon et al., 2001). These neurotoxins are secreted as 150-kDa proteins comprising a 100-kDa heavy chain and a 50-kDa light chain and are packed within a high-molecular-weight noncovalent, multimeric protein complex. The heavy chain facilitates binding, uptake, and release of the light chain into peripheral cholinergic nerve endings. Here the light chain, a zinc-dependent endopeptidase, targets certain molecules of the soluble N-ethylmaleimide-sensitive-factor attachment receptor (**SNARE**) complex, which is involved in vesicle fusion and release of neurotransmitter into the synaptic cleft. Proteolysis of the SNARE complex molecules leads to a block of neurotransmitter release, resulting in flaccid muscle paralysis (Simpson, 2004). Because of their extreme toxicity, the botulinum neurotoxins are classified by the Centers for Disease Control and Prevention in category A of bioterrorism agents.

In the current study, we show that high amounts of specific IgY antibodies can be obtained after immu-

nizing chickens with immobilized plant and microbial toxins. To optimize the cost:yield ratio we monitored the laying capacity and the total and specific IgY over an extended period. Interestingly, we observed a complex biological oscillation for the total IgY titer, with a shorter time period of about 7 d. Toxin-specific IgY were used for classical immunological detection methods and functional inhibition *in vitro* and *in vivo*.

MATERIALS AND METHODS

Birds

Sixteen-week-old chickens [ISA Brown and Lohmann Selected Leghorn (**LSL**), Spreenhagener Vermehrungsbetrieb für Legehennen GmbH, Bestensee, Germany] were kept in the Research Institutions of Experimental Medicine (Charité-Universitätsmedizin, Berlin, Germany) in individual cages, exclusively constructed for the maintenance of chickens (Ebeco, Castrop-Rauxel, Germany). Food (ssniff Legehühner-Zucht 1 and 2; ssniff Spezialitäten GmbH, Soest, Germany) and water were available *ad libitum*, and the chickens started laying eggs between 23 and 25 wk of age.

Eggs were collected daily, labeled, and stored at 4°C until they were further processed. All animal maintenance and experiments were performed in accordance with the guidelines of local authorities, Berlin (No. H0069/03).

Antigens

Ricin (approximately 98% purity) was kindly provided by U. Pfüller (University Witten/Herdecke, Germany), purified A- and B-chains of ricin were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and botulinum neurotoxins were purchased from Metabionics Inc. (Madison, WI). Botulinum neurotoxins A and B were covalently immobilized on beads as will be described elsewhere (D. Pauly, unpublished data). Ricin, BoNT/A beads, or BoNT/B beads were mixed in a ratio of 1:1 in Freund's complete adjuvant (**FCA**) or Freund's incomplete adjuvant (**FIA**; both from Sigma-Aldrich) for immunization.

Immunization

Chickens were immunized and boosted via the *i.m.* route (pectoralis muscle, left and right side) a total of 13 times over a 2-yr period, with intervals between 4 and 8 wk (for details, see Figure 1). The interval used was based on previous work that showed no demonstrable memory cells until at least 3 wk postimmunization (Pei and Collisson, 2005). The antigen concentration used was approximately 20 µg per injection (native ricin, immobilized BoNT/A, or BoNT/B). No more than 500 µL of antigen solution was injected per immuniza-

tion. Freund's complete adjuvant was used for the first immunization, and FIA was used for the subsequent booster injections.

IgY Extraction from Egg Yolk

The method for IgY purification was adapted from Polson et al. (1980). Briefly, the egg yolk was diluted 1:2 with sterile PBS (pH 7.4, Roche, Mannheim, Germany). For elimination of lipids and lipoprotein, 3.5% (wt/vol) polyethylene glycol (PEG) 6000 (Roth, Karlsruhe, Germany) was added. After gentle shaking followed by centrifugation ($10,000 \times g$ for 20 min at 4°C), the supernatant was decanted and solid PEG 6000 was added to a final concentration of 12% (wt/vol). This mixture was then centrifuged as above. The precipitate was dissolved in 10 mL of PBS, PEG was added to 12% (wt/vol), and the solution was centrifuged. Finally, the precipitate was dissolved in 1.2 mL of PBS, transferred into a microdialysis device (QuixSep, Roth, Germany) and dialyzed against PBS at 4°C.

The protein content (mg/mL) was measured photometrically at 280 nm and was calculated according to the Lambert-Beer law with an extinction coefficient of 1.33 for IgY.

ELISA

Nunc Maxisorp F96 microtiter plates (VWR International GmbH, Darmstadt, Germany) were coated with antigen (500 ng/mL) in PBS overnight at 4°C and then blocked for 1 h with blocking buffer of PBS containing 0.1% Tween-20 and 2% nonfat skimmed milk (Merck, Darmstadt, Germany). After washing, IgY dilutions were added (1:10,000 to 1:1,000,000 in blocking buffer) for 1 h and detected using biotin-labeled donkey anti-chicken IgY, streptavidin-horseradish peroxidase (both Dianova, Hamburg, Germany) and 3,3',5,5'-tetramethylbenzidine (Sigma).

Immunoblot

Ricin, the isolated A- and B-chains of ricin (ricin A and ricin B, respectively), BoNT/A, and BoNT/B were separated by 10% SDS-PAGE (under nonreducing conditions), and were transferred onto a polyvinylidene fluoride membrane (Invitrogen GmbH, Karlsruhe, Germany) using standard immunoblotting techniques. The membrane was blocked overnight at 4°C, and incubated with IgY (1:5,000 in blocking buffer) for 1 h. After washing, the membrane was probed with biotin-labeled donkey anti-chicken IgY for 30 min and was developed

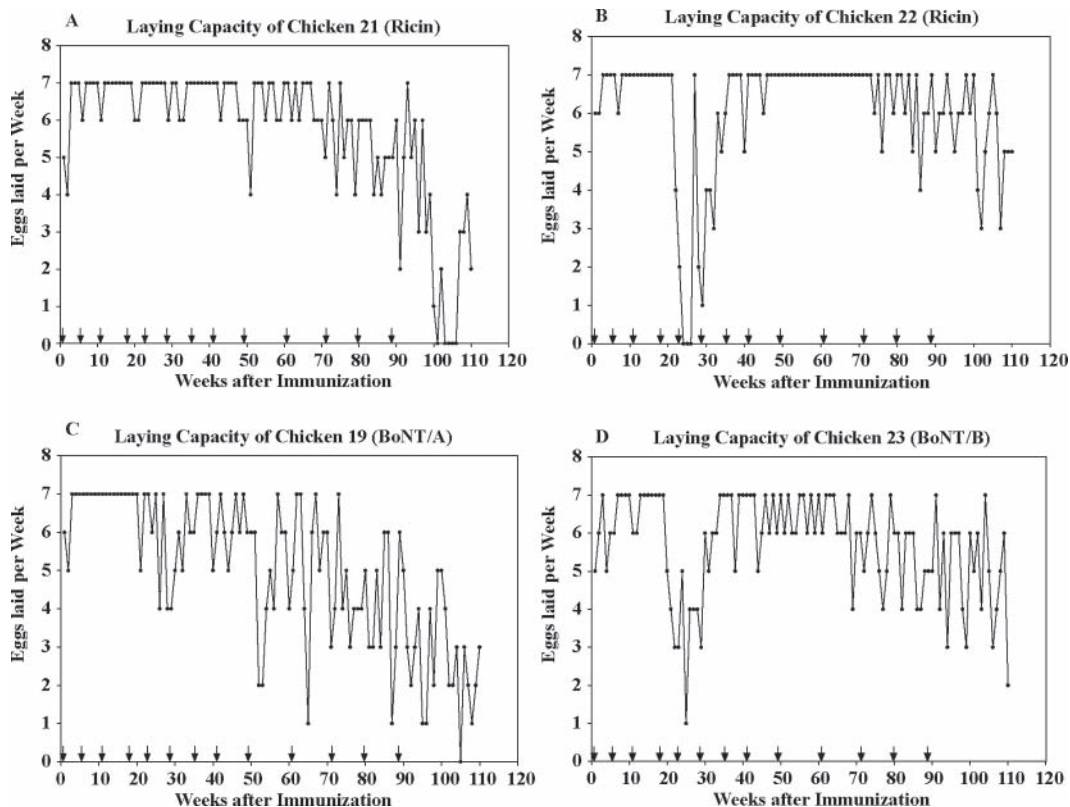


Figure 1. Egg-laying capacity of hens immunized to produce polyclonal IgY specific for different toxins during a 2-yr period. A) chicken 21 (ricin), B) chicken 22 (ricin), C) chicken 19 [botulinum neurotoxin (BoNT)/A], and D) chicken 23 (BoNT/B). The arrows indicate the time points of immunization.

Table 1. Statistics of egg-laying capacity of chickens immunized with ricin or botulinum neurotoxin (BoNT) antigens

Item	Chicken 21 (ricin)	Chicken 22 (ricin)	Chicken 19 (BoNT/A)	Chicken 23 (BoNT/B)
Eggs, n (total IgY, ¹ g), 2-yr period	625 (38)	626 (38)	545 (33)	608 (37)
Eggs, n (total IgY), yr 1	345 (20)	304 (16)	326 (18)	308 (17)
Percentage of maximum ²	95	84	90	85
Eggs, n (total IgY, g), yr 2	280 (18)	322 (21)	219 (15)	300 (20)
Percentage of maximum	77	88	60	82
Processed eggs, n	565	620	283	401

¹The amount of total IgY is calculated by multiplying the number of eggs by a mean value of 60 mg of protein per egg according to the data of Figure 2.

²The maximum possible egg number per year is calculated as 52 wk × 7 eggs = 364 eggs.

using alkaline phosphatase and CDP-Star (Perkin Elmer, Waltham, MA).

Block of Functional Activity In Vitro and In Vivo

Cytotoxicity Test. To determine the neutralizing capacity of IgY, a ricin cytotoxicity assay was performed, as described previously (McGuinness and Mantis, 2006). Briefly, 10⁴ African green monkey kidney (Vero) cells (ATCC, Manassas, VA) were cultured in 96-well plates for 18 h at 37°C. The antibody (chicken 22) was diluted stepwise (7.4 to 0.5 µg/mL; 60 µL/well) and 10 ng/mL ricin was added (60 µL/well). The mixture was incubated for 1.5 h at 37°C on a shaker. Vero cells were treated with ricin-IgY mixtures or ricin alone for 2 h, washed, and further incubated for 20 h. The viability of the Vero cells was determined using the CellTiter96 nonradioactive cell proliferation assay (Promega GmbH, Mannheim, Germany).

Mouse Assay. To test the neutralizing capacity of anti-ricin, anti-BoNT/A, and anti-BoNT/B IgY preparations, mice were challenged with the corresponding toxins that had been preincubated with IgY. A 5- to 10-fold excess of the LD₅₀ of the toxin was mixed with the indicated amount of IgY in PBS containing 1% BSA. After preincubation for 30 min at 37°C the solution was injected i.p. into female BALB/c mice and the animals were observed periodically over the next 10 d for signs of illness.

Software

GraphPad Prism (GraphPad, San Diego, CA) was used for preparing the figures. The chronobiological analysis was performed using Chronos-Fit, Version 1.05 (Zuther and Lemmer, 2004).

RESULTS

Immunization and Monitoring of Laying Capacity and IgY Content

To identify a suitable length of time that animals could be maintained, we monitored the number of eggs

laid per week by individual chickens immunized against different toxins. Chickens were immunized soon after they started to lay eggs, at around 22 wk of age. At this age they usually laid 7 eggs per week. Laying capacity started to decrease between 70 and 80 wk after the first immunization, corresponding to an age of nearly 2 yr. During the first year of the study the laying capacity of chickens 19 and 21 was close to the maximal number of eggs (300 to 320 eggs), whereas a 30 to 70% decrease in egg production was noted (2 to 5 eggs per week) during the second year (see Table 1). A different development was observed for chickens 22 and 23 because of a laying depression during the first year (see Figures 1B and 1D). This dramatic reduction in laying capacity was observed between wk 20 and 30 (corresponding with the summer months, July and August 2005) for no obvious reason, because all hens were kept in a single room within an air-conditioned animal facility.

In addition to the egg-laying capacity, the IgY concentration per egg is of major interest for antibody production. According to the extraction method used, about 90% of the protein is pure IgY, as tested by SDS-PAGE and Coomassie Brilliant Blue staining. As shown in Figure 2, the weekly mean IgY content (mean of chickens 19, 21, 22, and 23) per egg varied between 38 and 45 mg in the first 10 wk after primary immunization. Thereafter, the mean IgY content increased to values between 53 and 60 mg per egg, and this remained constant for approximately 1 yr. During the second year of immunization, the mean IgY content further increased to values up to 68 mg/egg. These data indicate that Leghorn hens can be used for approximately 2 yr to generate high yields of IgY. Although the number of eggs collected in the second year was lower than in the first year (Figure 1), the total IgY content was greater (Figure 2) and therefore compensated for the reduced egg number.

Periodic Fluctuation of the IgY Concentration

A macroscopic analysis of the course of IgY content of chicken 22 during a period of continuous egg laying (wk 36 to 82; corresponding to September 2005 to July 2006, see Figure 1B) seemed to point to a periodic fluctuation.

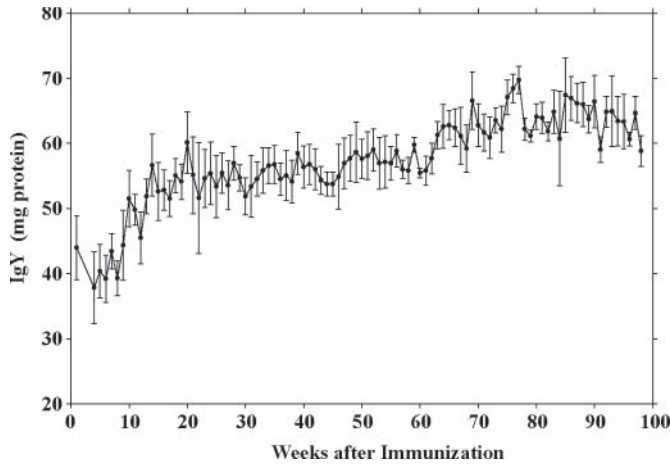


Figure 2. Development of the total IgY content during a 2-yr period. The weekly mean \pm standard deviation of IgY (mg) is given for chickens 19 to 23 ($n = 4$).

tuation of the IgY content, after processing the data by means of the moving average (Figure 3B). To test the assumption that the IgY content underwent a periodic fluctuation we analyzed the data using the Chronos-Fit program. A significant rhythm (rhythm 73.5%; $P < 0.001$) with a period length of approximately 60 d was identified (Figure 3C). Further detailed analysis of a part of the data (September 29 to October 15, 2005) showed a significant rhythm (96% rhythm; $P < 0.0001$) with a period length of 7 d (Figure 3D). Similar findings were obtained when analyzing the weekly mean IgY concentration of chickens 19, 21, and 23 (data not shown).

Antibody Characterization

Ricin Immunization. Two hens (21 and 22) were immunized with purified ricin and IgY antibodies were purified from individual eggs. The IgY were tested for

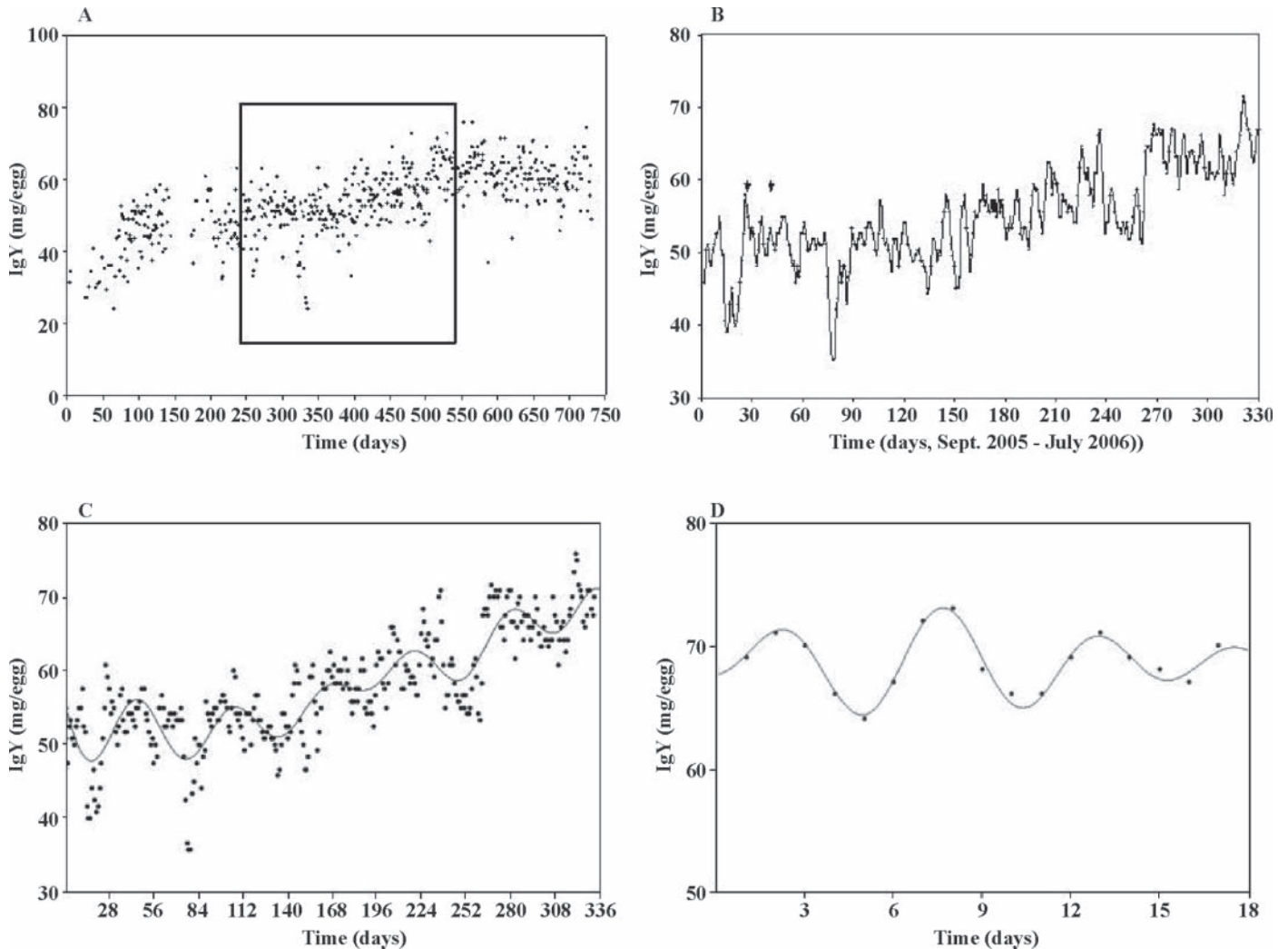


Figure 3. A) Time-dependent development of total IgY concentration of chicken 22 over 2 yr. The frame indicates the period further analyzed in B and C. B) Time-dependent oscillation of the daily mean IgY of chicken 22. The original data were processed by means of the moving average. The period corresponds to the period of maximum egg-laying in Figure 1B (wk 36 to 82). C) As in B, but the graph was analyzed using the Chronos-Fit program (significant period length around 60 d, 73% rhythm). D) As in B, but data were analyzed in more detail (period from September 19 to October 15) as indicated by arrowheads in Figure 3B (significant period length around 7 d, 96% rhythm).

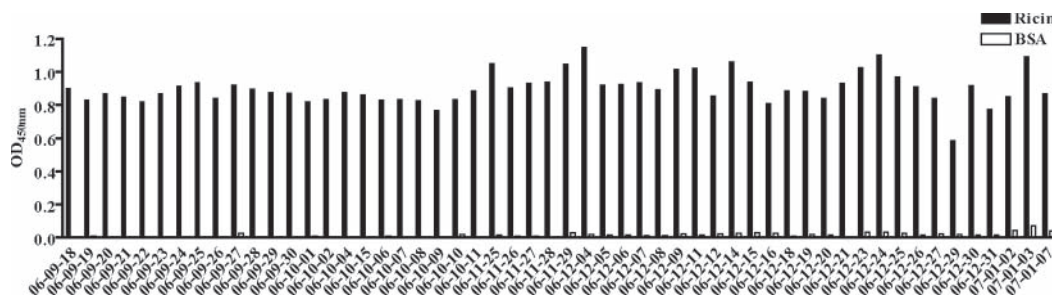


Figure 4. Results of ELISA using IgY isolated from individual eggs from chicken 22. Plates were coated with 500 ng/mL of ricin, blocked, and then probed with anti-ricin IgY (dilution of 1:1,000,000). Bound IgY was detected with biotinylated donkey anti-chicken IgG, streptavidin-HRP and 3,3',5,5'-tetramethylbenzidine. OD = optical density.

their ability to detect ricin in classical immunological assays. An ELISA against ricin revealed that a high antibody titer was obtained after the fifth immunization (chicken 21) or the third immunization (chicken 22), respectively, and the titer was stable for several weeks thereafter (see Table 2). Figure 4 shows the specific anti-ricin titer in individually processed eggs, starting 13 d after immunization on September 5, 2006, as measured in a 1:1,000,000 dilution by ELISA. Apart from slight variations, the antibody titer remained at a high level over 2 mo. Preparations of high antibody titer were pooled and analyzed further. As shown in Figure 5A, Western blot analysis showed that IgY originating from chickens 21 and 22 were able to detect ricin, as well as the highly homologous *Ricinus communis* agglutinin. Immunoglobulin Y from chicken 21 recognized both the purified A and B chains, respectively, and the IgY from chicken 22 detected specifically the catalytic ricin A chain.

BoNT Immunization. For the generation of antibodies against the botulinum neurotoxins, 1 chicken was immunized with immobilized BoNT/A and 2 with immobilized BoNT/B. In the case of BoNT/B, 1 chicken did not produce a significant antibody titer after 8 immunizations and was therefore not included in the study. Experimental testing of the purified antibodies using ELISA assays showed that chicken 19 (BoNT/A) and chicken 23 (BoNT/B) developed a titer against the corresponding toxins after 6 and 2 immunizations, respectively (Table 2). Figure 5B shows an immunoblot of purified BoNT/A and BoNT/B, probed with the respective antibodies. Immunoglobulin Y from chicken 19 recognized the 100-kDa heavy chain, the 50-kDa light chain of BoNT/A, and also the whole protein at 150 kDa. Antibodies from chicken 23 (BoNT/B) detected

predominantly the heavy chain and the holo-toxin (Figure 5B). As well as detecting denatured toxin in immunoblot experiments, both anti-botulinum toxin antibodies (chickens 19 and 23) and anti-ricin antibodies (chickens 21 and 22) also recognized the native toxin in sandwich ELISA experiments (D. Pauly, unpublished data).

Neutralizing Capacity. An important goal for the generation of avian IgY was to obtain antibodies that were able to block the functional activity of the respective toxins. In the case of ricin, we used a cytotoxicity assay to test if the anti-ricin IgY were able to prevent the toxic activity in vitro. Both IgY from chickens 21 and 22 were found to neutralize the toxic effect of ricin on Vero cells (Figure 6A). Our data showed that between 3.7 and 7.4 $\mu\text{g/mL}$ of IgY (from chicken 22) completely prevented ricin-induced cell death. In contrast, Vero cells that were treated with the same concentration of ricin (10 ng/mL) in the presence of an irrelevant IgY preparation or without any IgY antibody subsequently died (Figure 6A and data not shown). In addition, blocking capacity was tested in vivo with ricin preincubated with IgY from chicken 22, or an irrelevant control IgY. All animals that received ricin in combination with IgY from chicken 22 survived, whereas the control mice died within 24 h of injection (Figure 6B).

In the case of anti-BoNT/A and anti-BoNT/B IgY, we tested the neutralizing capability of the antibodies by using a classical mouse assay: BoNT/A and BoNT/B were preincubated with the IgY preparation of chicken 29 or chicken 23, respectively, or with an irrelevant control IgY. Mice that were injected with toxin in combination with the corresponding anti-toxin IgY survived, whereas the control animals died within the first 2 d (Figure 6C and D). In summary, IgY specific

Table 2. Development of antibody (Ab) titer in chickens immunized with ricin or botulinum neurotoxins (BoNT) antigens

Chicken	Antigen	Eggs tested, n	Immunizations, n	First Ab detection (after x immunizations)	Stable Ab titer (after x immunizations)
21	Ricin	565	13	2	5
22	Ricin	620	13	2	3
19	BoNT/A	283	13	6	11
23	BoNT/B	401	13	2	4

for ricin, BoNT/A, or BoNT/B were able to neutralize the functional activity of their corresponding toxin *in vivo* and thus may have potential therapeutic value when characterized further in animal models.

DISCUSSION

In the current work, we monitored the egg-laying capacity of chickens immunized with different toxins over a 2-yr period. We compiled information on total IgY content and specific anti-toxin titer in individually prepared eggs to optimize the cost:yield ratio. Our data show that chickens can be used for up to 2 yr for the generation of high amounts of IgY and that the observed reduction in laying capacity during the second year is compensated by a greater amount of total IgY per egg. Thus, antibody recovery remains high even after prolonged immunizations. The IgY concentration follows clear age-dependent kinetics, with around 30 mg/egg

at the beginning of egg-laying and up to 80 mg/egg during the second year of the study. The IgY concentration, however, depends considerably on the extraction method used, as well as the weight of the egg, the breed of the chicken, and the method used for quantification of the IgY (Bizhanov and Vyshniauskis, 2000; Stålberg and Larsson, 2001; De Meulenaer and Huyghebaert, 2001; Carlander et al., 2003; Kitaguchi et al., 2008). There are considerable differences in recovery rates of the IgY yield for the Polson method ranging between 15 and 150 mg/egg, and this is in accordance with the 30 to 80 mg of IgY per egg found in our study (Altschuh et al., 1984; Hassl and Aspöck, 1988; Gassmann et al., 1990; Bizhanov and Vyshniauskis, 2000; Stålberg and Larsson, 2001). Not all of the IgY present in the yolk is purified with the method described by Polson, and more efficient methods are reported (Stålberg and Larsson, 2001). Nevertheless, IgY of high purity is obtained with ease at low cost.

Early studies of plant and microbial toxins have established that chickens tend to be more resistant against toxins than other species (Kitasato, 1891; Klemperer, 1893; van Ermengem, 1897; Behring, 1900; Mießner and Rewald, 1909; Bengtson, 1924). Depending on the toxin, minuscule amounts of the native molecule are poisonous, thereby limiting the use of native toxin for immunization. When we analyzed the number of eggs laid before and after immunization (Figure 1), we observed no obvious influence on laying capacity after immunization with immobilized native toxins. This was found even when amounts several fold above the lethal dose of the free toxin were used. In particular, chicken 22 (immunized with ricin) was producing at the maximum level (7 eggs/wk) during wk 46 to 73, despite 3 immunizations during that time (Figure 1B). We also compared the total number of eggs per year (as shown in Table 1), with the data available. The performance of ISA Brown is approximately 84 to 88% (corresponding to 308 to 320 eggs/yr), and that of LSL is approximately 83 to 85% (corresponding to 301 to 311 eggs/yr) (Abrahamsson and Tauson, 1998; Li et al., 1998; Hendrix Genetics, 2006; Lohmann Tierzucht GmbH, 2008). In our study we used ISA Brown (chickens 19, 21, and 23) and LSL (chicken 22), which produced 304 to 345 eggs in the first year (Table 1). This is in accordance with the above findings in the literature, but does not account for different housing conditions. The absence of any obvious influence on the laying performance indicates that neither the toxin composition used for immunization nor the FIA had a major impact on the birds' performance. Although our data do not indicate any deleterious effects of the immunization, we cannot completely exclude such an effect because of the small number of birds and the lack of controls in this study. Furthermore, the influence of FCA and FIA as adjuvants for immunization has been the cause of controversial discussion in the previous literature. Bollen and Hau (1999) reported that FCA and FIA caused a significant depression in egg-laying frequency,

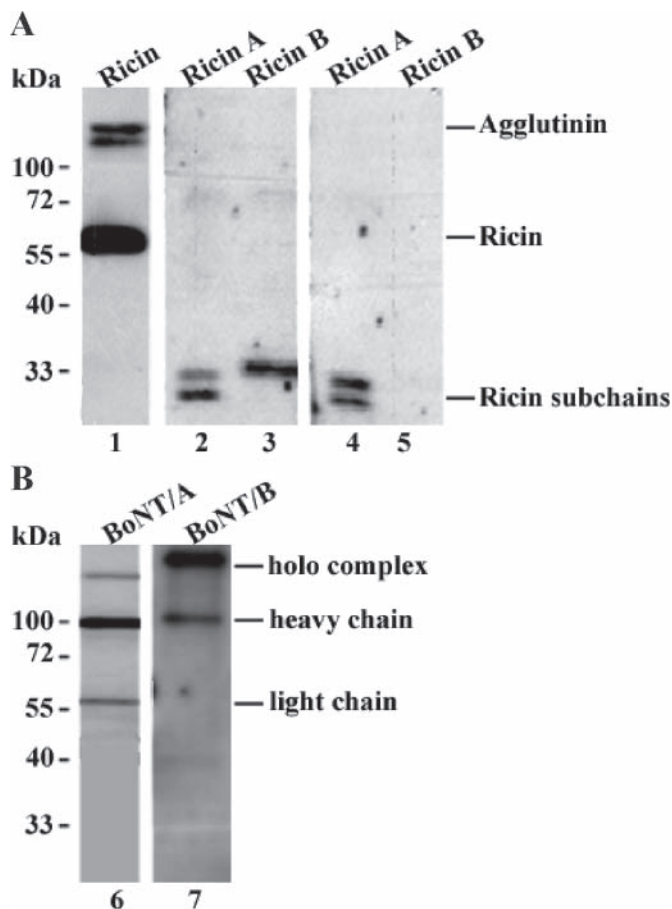


Figure 5. Immunoblot detection of ricin, botulinum neurotoxin (BoNT)/A and BoNT/B using anti-toxin IgY. Lane 1 = 25 ng of ricin (RCA60); lane 2 and 4 = 25 ng of ricin A-chain; lane 3 and 5 = 25 ng of ricin B-chain; lane 6 = 25 ng of BoNT/A; lane 7 = 25 ng of BoNT/B. Proteins were separated on a 10% SDS-PAGE under nonreducing conditions and transferred to a polyvinylidene fluoride membrane. The membrane was divided and probed with dilutions of IgY (1:5,000) from different chickens: lanes 1, 4, and 5 with IgY from chicken 22; lanes 2 and 3 with IgY from chicken 21; lane 6 with IgY from chicken 19; and lane 7 with IgY from chicken 23. The identity of the detected proteins is indicated on the right side.

whereas others found only a minor or transient effect or even failed to notice an influence (Erhard et al., 1997; Schade et al., 2005). It is also possible that these results reflect differences in FCA and FIA sensitivity between the breeds used in the different studies.

A result rather by chance was our observation that the development of total IgY during the 2-yr period appeared to follow complex significant biological oscillations. The finding was evident both for the mean value of the 4 chickens and for the individual chickens (e.g., chicken 22, Figure 3). Interestingly, the smallest rhythm observed was a 7-d one, the so-called circaseptan rhythm, confirming earlier observations by (Schade et al., 2001). Circaseptan (or weekly) rhythms have been noted and discussed in the literature; for example, in connection with cardiac mortality, blood pressure, excretion of steroids, as well as in the immune system (Cornelissen et al., 1993; Labrecque et al., 1995; Haus and Smolensky, 1999; Halberg et al., 2003). We interpreted the 60-d rhythm (observed in Figure 3C) as an approximate multiple of 7, a finding that was also reported for several components of the immune sys-

tem (Haus and Smolensky, 1999). At present, it is not clear what the biological meaning of the circaseptan rhythm in the IgY content may be. On the one hand, the hen has to ensure a constant IgY concentration to supply all of the offspring with an optimal amount of IgY (Carlander et al., 2001). On the other hand, however, the fate of more than 90% of yolk IgY is uncertain (Kowalczyk et al., 1985). It has been estimated that only around 10% of the yolk IgY has importance in the passive immunity of the newly hatched chickens. Thus, it seems that a biological rhythm of IgY would not be a risk for the passive immunity of the hen's offspring. Generally, it would be interesting to understand at the molecular level the synthetic activity of chicken B lymphocytes, in view of a rhythmic production of different amounts of IgY.

In addition to differences in IgY production between several breeds of chickens and differences in the extraction procedures used, the observed fluctuation of the IgY content may be an additional reason for the different IgY concentrations in the egg yolk observed by various authors (Bizhanov and Vyshniauskis, 2000;

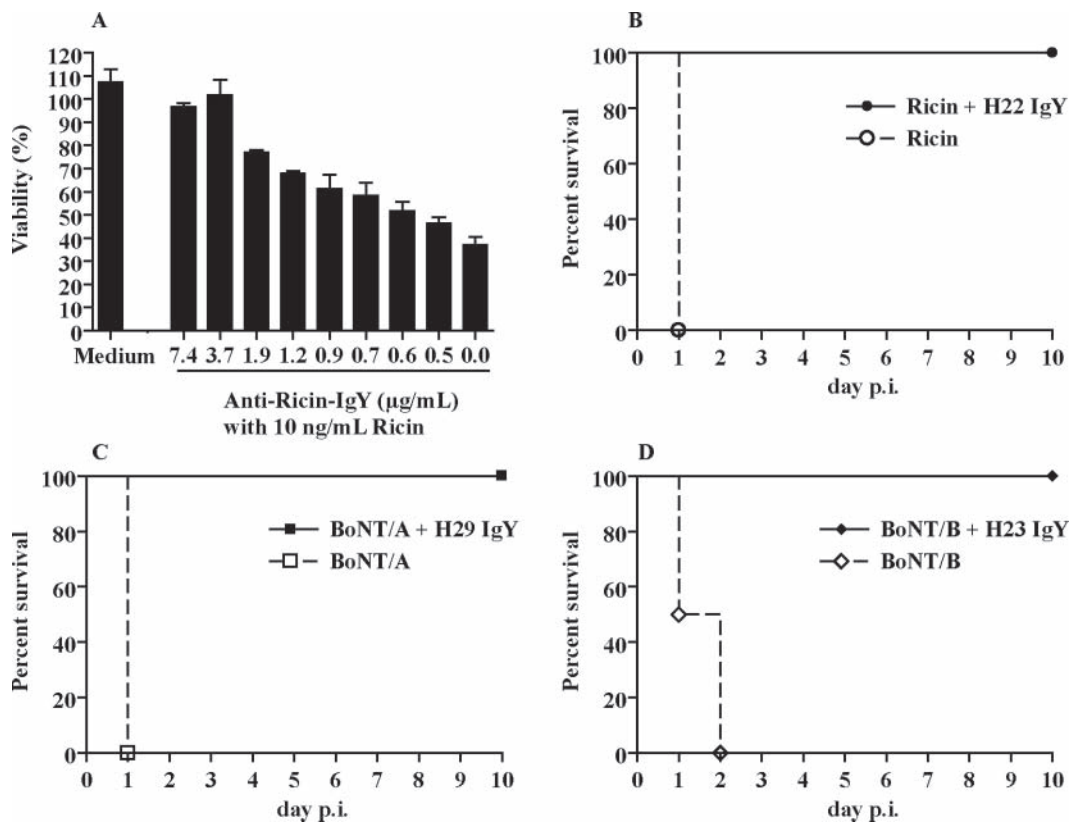


Figure 6. Neutralizing capacity of anti-toxin IgY. A) Anti-ricin IgY neutralizes ricin toxicity in vitro. Anti-ricin IgY (example shown is chicken 22) in different concentrations, were premixed with 10 ng/mL of ricin and incubated on Vero cells, as described in Materials and Methods. As controls, viable cells without ricin treatment (medium) and cells treated with ricin in the absence of blocking antibodies are shown. B) Anti-ricin IgY block toxicity of ricin in vivo. Five BALB/c mice per group were challenged i.p. with 200 µL of the mixture containing 1 µg ricin [$5 \times 50\%$ lethal dose (LD₅₀)] and 200 µg of IgY from either chicken 22 (H22, closed symbols) or an irrelevant control animal (open symbols). Animals were monitored over 10 d postinjection. Survival is shown as a Kaplan-Meier plot. C) Anti-botulinum neurotoxin (BoNT)/A IgY blocks BoNT/A toxicity in vivo. Five BALB/c mice per group were challenged i.p. with 200 µL of the mixture containing 100 pg of BoNT/A ($10 \times$ LD₅₀) and 100 µg of IgY from chicken 29 (H29; closed symbols), or from a control animal (open symbols). Animals were monitored over the next 10 d. Survival is shown as a Kaplan-Meier plot. D) Anti-BoNT/B IgY blocks BoNT/B toxicity in vivo. Mice were challenged i.p. with 200 µL of the mixture containing 50 pg of BoNT/B ($10 \times$ LD₅₀) and 100 µg of IgY from chicken 23 (H23; closed symbols; 5 animals) or 100 µg of irrelevant IgY (open symbols; 2 animals). Animals were monitored over the next 10 d and the survival is shown as a Kaplan-Meier plot.

Stålberg and Larsson, 2001; Carlander et al., 2003). According to our results the difference between maximum and minimum IgY levels during time can be up to 40%.

When we tested the polyclonal chicken antibodies in classical immunological and functional assays we found that they not only recognized the denatured toxin (Figure 5), but also performed well in the detection of the native toxins. For example, this was observed in sandwich ELISA assays in combination with monoclonal antibodies. In line with the detection of the native toxin via ELISA, the chicken antibodies were able to neutralize ricin (in an in vitro cell culture cytotoxicity assay), and BoNT/A or BoNT/B toxicity (in an in vivo mouse bioassay, Figure 6). Neutralizing capacities of chicken IgY have been reported by several researchers. In a similar approach, Lemley and coworkers raised an avian antitoxin against ricin with blocking efficacy by immunizing chickens with 0.5 mg of ricin toxoid (Lemley et al., 1995). For a lethal staphylococcal enterotoxin B aerosol exposure, LeClaire and colleagues found that postexposure treatment of monkeys with chicken IgY prevented death (LeClaire et al., 2002). Others have similarly generated chicken antitoxins against *Clostridium difficile* toxins, rabies, or viper venom (Kink and Williams, 1998; Maya Devi et al., 2002; Motoi et al., 2005).

Conclusions

In summary, our data are useful to optimize the cost:yield ratio when producing quantitative amounts of polyclonal IgY. In good accordance with previous reports, our data on chicken egg-laying performance show that the number of eggs produced declines from wk 80 (around 2 yr of age) postimmunization (Figure 1 and Table 1). Taking into account the 30% increase in IgY content per egg (50 to 65 mg/egg at the end of the second year) and comparing it to the decline in egg numbers, a maintenance period of 2 yr was determined as the period that allows a relatively stable yield of IgY. This, however, may vary between individuals. Insofar as the individual performance of immunized hens is already monitored, the maintenance period may be extended until bird performance decreases to about 4 eggs per week. Keeping hens for a second year extends the time for antibody production, thus doubling the yield of specific IgY antibodies.

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