出國報告（出國類別：研習）

研習雞種原保存技術

服務機關：行政院農業委員會畜產試驗所
姓名職稱：林秀蓮助理研究員
派赴國家：法國
出國期間：102年7月14日至102年7月27日
報告日期：101年9月26日

目　錄

頁次

壹、 摘要

台灣擁有優良種雞品種，且因地處亞熱帶地區，種雞長期於此環境下已發展出耐熱特性之競爭優勢，進軍亞太市場除可拓展活體外銷外，推廣精液產品爲高效率且經濟之方式。然國內對於精液低溫保存之研究，包括軟硬體設備在內，多以家畜作爲主要使用對象作設計，致使用於鳥禽類精液凍存之相關技術及設備明顯不足而亟待加強。有鑒於此，本出國計畫由畜產試驗所研究人員於 102 年 7 月 14 日至 27 日期間赴法國研習種雞冷凍精液製作關鍵技術，並參觀法國國家級家禽種原庫及種雞保種場。研習地點包括法國卡蘇生技公司（I.M.V. Technologies France）、法國國家農業研究院圖爾分院（INRA-Tours）及法國里昂大學幹細胞與大腦研究所（Stem-cell and Brain Research Institute, SBRI），因而有機會了解目前法國在冷凍生物學領域的各項發展及近期主要執行中的任務計畫，如母雞生殖道構造組成與其受精持續力長或短之關聯性之研究、母雞生殖道及雞精液蛋白質體之研究、家禽始基生殖細胞之應用等。在與 INRA-Tours 研究中心之 Dr. Pascal Mermillod、Dr. Elisabeth Blesbois 與 Dr. Bertrand Pain 討論後，三位專家以自身經驗提出多項寶貴之意見及建議，並達成雙方未來持續相互合作之意願與共識。返國後借鏡此行所習得之法國經驗，希望可加速國內雞精液凍存技術發展，提升國內種雞產業水準，同時提高雞種原保存效率以達到維護種原多樣性之目的。

貳、 目的

爲保存動物遺傳資源，除可藉由擴大動物族群數目達成，保存生殖細胞亦爲有效且經濟之方法。惟母禽生殖細胞之保存有其先天限制，無法似哺乳類般取其卵母細胞或胚來進行種原保存，故自雄禽取其精細胞凍存仍爲目前保存鳥禽類遺傳資源最有效之方法。然而，國內對於精液低溫保存之研究，包括軟硬體設備在內，多以家畜作爲主要使用對象作設計，致使用於鳥禽類精液凍存之相關技術及設備明顯不足而亟待加強。台灣擁有優良種雞，且因地處亞熱帶地區，種雞長期於此環境下已發展出耐熱特性之競爭優勢，倘能發展穩定種雞精液凍存技術，製備優良種雞之冷凍精液輸出國際市場，將可提升我國種雞知名度並增加業者所得，爲實現我國成爲「亞太種畜禽中心」之重要一環。

法國國家農業研究院（INRA）爲歐陸最先進的農業研究機關，其圖爾（Tours）分院研究團隊針對各類禽畜繁殖學研究均有非常卓越之成果。此外，該院於 2003 年在法國農業部指導下成立國家級種原中心，其家禽種原庫之主持人 Dr. Elisabeth Blesbois 爲此次邀訪專家之一。Dr. Elisabeth Blesbois 及其研究團隊，長期以來對於種雞精液凍存有深入之研究，該實驗室亦具備完善的儀器設備評估精液品質性能，對於國內種雞冷凍稀釋液配方開發及冷凍解凍流程建立所需之各項技術必能給予有效建議，且其管理法國家禽種原庫之寶貴經驗必可做爲台灣畜產種原庫管理參考。

因此，本出國研習計畫之主要目的有二，其一爲加強台法合作關係，期能建立國際合作團隊；其二則希望透過赴法研習及邀請法方專家訪台針對雞精液凍存技術及家禽種原庫建置與管理作深入探討，除有利將來相關科技計畫執行，更有助於提升國內種雞產業水平。

參、 過程

赴法研習行程

(一) 研習行程

表 1. 林秀蓮赴法研習行程

日期			地點	內容	接待專家
月	日	星期			
7	14	日	台北	搭機至法國	---
	15	一	巴黎（IMV 公司）	抵達法國戴高樂機場	Misha Savic
	16	二		參觀 I.M.V.技術部門及生產線	
	17	三	圖爾（INRA）	Slides 準備（Pascal's lab）	---
	18	四		家畜生殖生理實驗室	Pascal Mermillod
	19	五		家畜試驗場	
	20	六		參訪資料彙整	
	21	日		Slides 準備（Elisabethe's lab）	
	22	一		國家家禽種原庫	Elisabeth Blesbois
	23	二		家禽冷凍精液研習	
	24	三	里昂（里昂大學）	雌禽生殖細胞保存	Bertrand Pain
	25	四	巴黎	參訪資料彙整	
	26	五	巴黎	搭機返台	---
	27	六	台北	抵達桃園機場	---

(二) 研習重點

1. 法國卡蘇生技公司（I.M.V. Technologies France）

在 Dr. Misha Savic 安排之下，先來到 I.M.V. Technologies 之一子公司 Cryo Bio System（Groupe I.M.V. Technologies）參觀，該公司主要任務爲研發冷凍生物學之

相關設備產品，包括各式冷凍麥管（圖 1）及自動充填設備（DIVA, 圖 2）等。DIVA 具有全自動化、品質管控、生物安全、樣品可追溯及錯誤管理系統等各項優點，每小時可充填 168 支冷凍麥管，對高度生物安全性及高效率要求之國家級種原庫，甚至是全球種原庫而言，實是品質控管上一大利器。

圖 1. CBS 高生物安全冷凍麥管。

圖 2. DIVA 麥管自動充填設備。

緊接著，在 Dr. Misha Savic 帶領下，繼續來到位於法國西北部諾曼地 L'agle 區的 I.M.V.本部參觀（圖 3 及 4）。抵達後先與服務於精液品質檢測技術研發部門的 Dr. Ludivine Chevrier 討論 GUAVA Eascyte 用以檢測雞精液品質上所面臨的各種問題（附錄一），期藉由討論結果於返國後將檢測條件設定稍作適當調整，使各項精液檢測結果更加精準。接著便在 Dr. Misha Savic 引導下參觀 I.M.V.各項產品生產線，了解該公司廠區如何運作，以維持各項產品品質管理。

圖 3. 計畫執行人與 Misha 合影於 I.M.V.公司。

圖 4. 與 I.M.V 生產部門負責人合影。

2. 法國國家農業研究院家畜生殖生理中心及家畜試驗場

來到 INRA 位於圖爾的 Nouzilly 研究中心，首先由 Dr. Pascal Mermillod 引導

參觀其研究團隊所屬實驗室（圖5），該團隊擁有純熟的山羊腹腔鏡授精、採胚及胚移置等人工生殖技術，其在山羊卵子體外成熟與體外受精等技術之發展領先全球，目前致力於研究複製山羊懷孕期胚胎發育與分子生物學的研究，希望有助於解決複製胚懷孕早期損失的問題。接著與研究人員 Dr. Nadime Gerard 討論執行之研究計畫，自 2012 年起 Dr. Nadime Gerard 便開始著手進行研究母雞生殖道構造組成與其受精持續力長或短之關聯性。研究重點主要分爲二大主軸（附錄二），其一爲探討母雞生殖道組織學構造，如陰道壁上的精子貯存小管（sperm storage tubule, SST）數量或大小與精子間之交互作用；其二爲蛋白質研究，藉由抽取 SST 內之液體分析其蛋白質組成，希望可以找出和精子有關之蛋白質標的物質未來進一步加以利用。討論結束後，緊接著來到中心內的家畜試驗場，其所飼養之動物包括有馬、綿羊、山羊、豬及牛。馬主要用以研究超音波採卵、精液冷凍製備、胚體外成熟與冷凍胚移置等；綿羊、山羊與豬則用以供卵或供胚，進行動物複製與基因轉殖相關研究；而牛則因爲考量研究成本，已不再執行動物複製與基因轉殖計畫，但仍提供做爲胚移置和冷凍，以及營養標準需求精準化研究之用。來此參觀令我印象最深且感觸之處，是現場工作人員善待動物的方式，以山羊爲例，畜舍內提供了多樣化的玩具（包括滾動的刷子跟球）（圖 6），讓羊群可以有足夠的運動與休閒；而幫馬施行人工授精時，授精夾欄可以同時容納母馬與哺乳中的小馬（圖

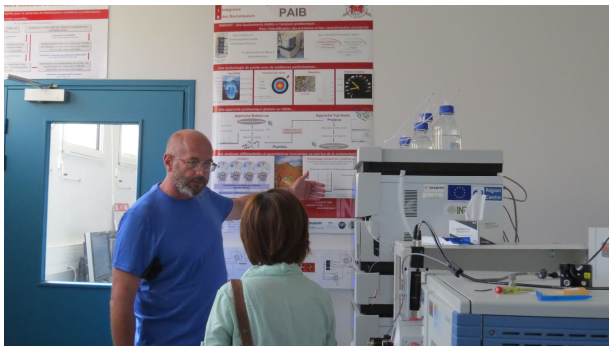


圖 5. Dr. Pascal Mermillod 引領參觀研究團隊及實驗室。


圖 6. 飼養於 INRA-Tours 之試驗動物有玩具可供使用。

7)，因爲母馬唯有跟小馬如影隨形才可以安心。重視動物福祉，除了是人道管理所必須，以科學論證而言，動物擁有健康快樂的身心靈，所獲得的試驗結果才是準確可靠的。

圖 7. 母馬在小馬陪伴之下接受人工授精。

3. 法國國家農業研究院家禽生殖生理中心及家禽種原庫

Dr. Elisabeth Blesbois 爲法國國家級家禽種原庫主持人，亦爲歐盟鳥類生物多樣性執行團隊一員，自 2010 年即與匈牙利 Dr. Judit Barna 共同主導 Cryobirds-Develpment of avian reproductive biotechnologies for the management of genetic biodiversity 計畫（附錄三），且其於 Nouzilly 研究中心之研究團隊長期以來對於家禽精液凍存有深入研究，所屬實驗室亦具備完善的儀器設備，該團隊於全球家禽精液凍存領域具有前導地位。與 Dr. Elisabeth Blesbois 會面後（圖 8），先針對目前製作種雞冷凍精液以及人工授精所面臨之結果與各項疑問作一簡單報告與討論（附錄四），她給了如下的建議：(1) 採精時的環境溫度影響甚鉅，溫度若高於 20℃必須於採精後立刻作稀釋。(2) 施行人工授精時，精子數目爲成敗關鍵必須精準控制，每次授精至少注入 $3X10^8$ sperms。(3) 確認母雞生殖道已準備好接受授精，可使用定量微吸管確保注入精液完全進入生殖道。爲讓我了解其實驗室標準作業流程，Dr. Elisabeth Blesbois 特別安排我進入種雞舍，從採精、稀釋、精液品質分析、一直到凍存過程，逐步觀摩學習比較異同，以作爲返國後參考依據，希望能提升目前製作種雞凍精之水準與效率。種雞舍負責人爲 Mr. David Gourichon，他先示範的是採精方式（圖 9），與國內傳統採精瓶相較，其所使用的分吸管採精瓶可收集到不被糞便污染的精液（圖 10）。之後 Mr. David Gourichon 示範了人工授精的方法，在施行授精前須先充分按摩母雞腹部，而所使用的定量微吸管可準確控制進入母雞生

圖 8. 與 Dr. Elisabeth Blesbois 會面進行討論。

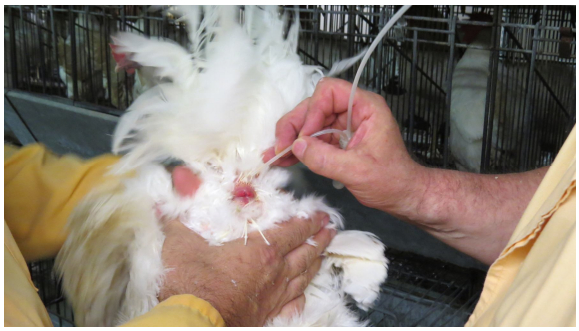


圖 9. Mr. David Gourichon 示範雞採精。

圖 10. 以分吸管採精瓶採得精液。

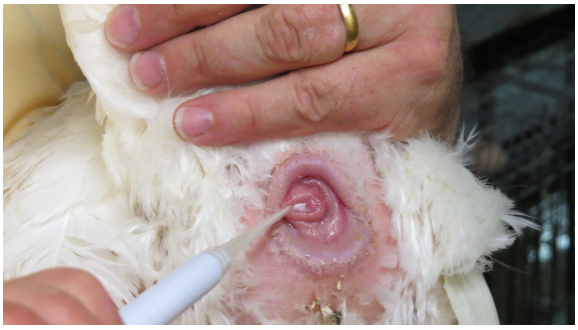


圖 11. 以微量吸管施行雞人工授精。

殖道精液量（圖 11）。David 更細心地提醒了注精時要注意授精管尖端，若有刺傷母雞陰道之虞，務必想辦法改變注精管末端形狀成平滑。完成示範操作後，Mr.David Gourichon 引導參觀位於該中心內之異地備份保種雞舍，該舍保存了來自法國各地特色雞種（圖 12）（附錄五）。完成種雞舍及異地保種雞舍參觀後隨即返回精液凍存實驗室，由 Miss Esabell 示範冷凍精液製作流程，並仔細解說了各項檢測精液性狀品質的技術與設備，包括精子活力（Mass Motility）、存活率、頭帽完整性及獲能反應。種蛋受精率取決於精子活力（Mass Motility）、存活率、頭帽完整性及獲能反應。種蛋受精率取決於精子活力，特別是前進（progressive）游動精子所占之比例爲受精率重要參考依據。因此 Miss Esabell 示範了如何操作 CASA（Computer Assisted Semen Analysis）測得各項活力數據（圖 13），並一一解說各數據所代表之意涵（圖 14）。

圖 12. INRA-Tours 研究中心保種場之各地法國土雞。

圖 13. Esabell 示範以 CASA 檢測雞精液品質。

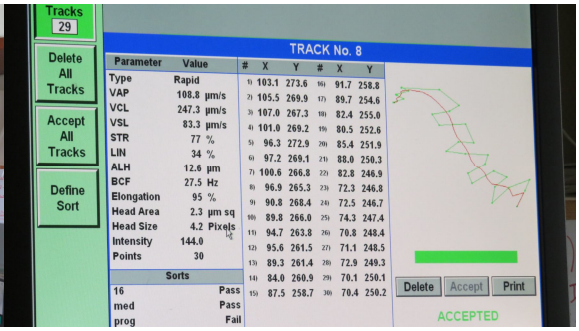


圖 14. CASA 所得之精液活力各項性狀數據。

4. 法國里昂大學幹細胞與大腦研究所

Dr. Bertrand Pain 對家禽幹細胞之研究獨步領先全球，其早在 1996 年便成功自雞胚中分離出胚葉細胞，並於培養液中添加生長因子進行體外培養，而後將培養七天之胚葉細胞注入雞胚後，成功得到嵌合體雞。此性腺嵌合雞保有原雞胚之遺傳物質，是雞種原保存除了活體保種及精液凍存外之另一種新方式。Dr. Bertrand Pain 介紹了其研究團隊所屬成員及實驗室，並表示高度意願未來可透過台法跨國合作計畫取得進一步合作的機會。

肆、 心得與建議

此次能有機會赴法國觀摩研習，在這個歐陸最先進的農業研究機構－INRA，所見識學習到的包括從上，政府部門願意大量投入研究經費，跟隨世界潮流科技，建置新穎儀器設備，提昇機構內的研究質能，讓研究人員有後盾可以充分發揮所長；從中，機構編組得宜，使人力充分運用並且各司所職，技術人員各有專精；從下，在重視動物福祉的飼養下，試驗動物擁有健康快樂的身心靈，使得科技計畫得以順利執行，並且獲得可靠的試驗結果。在在都是 INRA 能夠產出兼具高質與多量的試驗成果，使其聞名全球的要素。本出國計畫研習主題爲雞種原保存技術，除希冀有機會得以參訪法國家禽種原異地備份保種場以作爲回國後將來思考保種策略之參考依據外，更渴知的部分爲法國對於家禽生殖細胞保存之各項關鍵技術。一路參訪研習下，發現研究人員在各項細節都是非常小心謹慎的，包括提供給動物的飼養環境是舒適的、採集精液的方式則是盡量避免外源性污染，以求獲得最高品質精液，盡其所能改善每一階段所可能對精液造成的負面影響。而爲了清楚透視精子各項性能表現所建置的試驗設備，特別是與受精率緊密相關的精子活動力觀察系統－CASA，是本次最希望帶回來的技術，也期望未來透過科技計畫研提可以順利取得研究經費，在本實驗室建置該系統設備。除了實質上技術的研習外，在研究精神態度上的學習亦是收穫良多，此次在與法國專家接觸的過程中，發現他們是虛心的，廣泛的閱讀期刊以作爲試驗上的靈感來源，而且非常樂意分享研究成果，這些都是非常值得學習與效法的。國際合作計畫就像是蝴蝶效應一樣，在與國外專家討論過程當中，可以學習光是閱讀期刊所學習不來的部分，一直到建立良好默契，互相交換彼此的見解與概念，刺激雙方的試驗靈感。故建議國際合作計畫在經費與人員數量上多予支持，除可開闊研究人員的視野，刺激研究人員的想法，更能在國外專家的帶領之下，迅速提升國內研究水平與產業水準。

附錄一 20130712 (Wed)

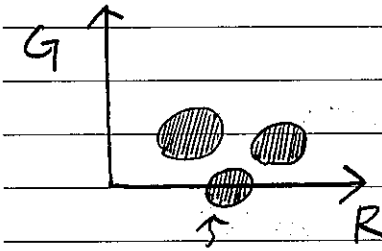
NO
DATE

IMV in FR

Suspecious Data

how to Improve the results

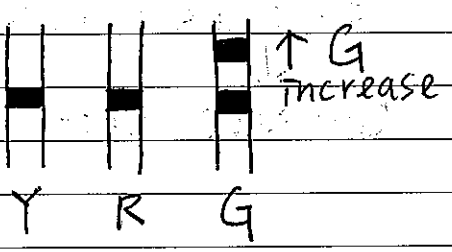
1. Adjust the Gate

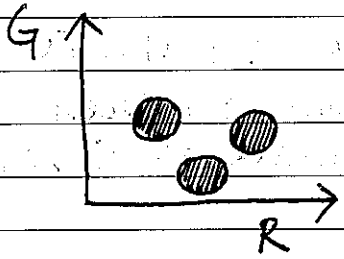


< Before Adjustment >

which is not good b/c stick some samples.

Adjustment





< After Adjustment >

✡ Centralized is needed !

May the joy and happiness around you today and always.

NO
DATE

How to save settings (Go Settings)

C: Programe file (Method 1)

↓

Guava Soft

↓

Cytofile

↓

Settings if overwrite

↓

change settings since now

<another method>

save as (can be saved anywhere)

↓

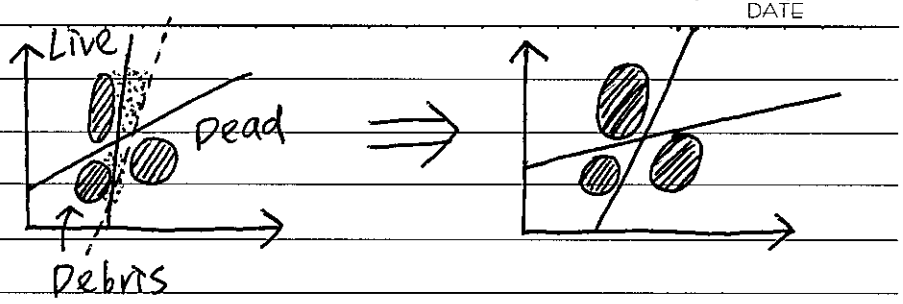
Retrieve Settings

2. When finish analyzing, check the plot

change the 1st plot, apply current settings to select samples, then check one by one to make all sure!

Much Better!

DATE



✡ Tips

- Concentration < 500 cells/ul
  200-500 is good.

– Setting analyze 5000 cells, so if conc is 500 → it takes around 10 secs to finish
If it takes too long to finish, which means the Conc is too low.

– Warnning (Red) = the Conc is too high,
if Conc < 550, it's still not big problem
if Conc 1000, very very big problem.

✡ About the film Theme.

Collect Semen from local farms → Lab → Cytoflow analyze semen → how apply the machine.

May the joy and happiness around you today and always.

附錄二 20130718.

NO.
DATE

INRA

✡ SST (Sperm Storage Tubule)

Researcher: Nadime Gerard
≈ 1 year on this topic
Email: nadime.gerard@tours.inra.fr

① Histology study
tubule × sperm Interaction
Ex. SST numbers, sizes ...

② Protomic study
Special protein
Sperm specific protein → relative markers.
↑
tubule fluid
~~from long-lasting and short-lasting~~
sperm storage strain hens,

David Gourichon.

May the joy and happiness around you today and always.

附錄三

Presentation cryobirds WPC 2012

Fri, 09/07/2012 - 15:08 | Elisabeth Blesbois

DEVELOPMENT OF AVIAN REPRODUCTIVE BIOTECHNOLOGIES FOR THE MANAGEMENT OF GENETIC DIVERSITY: CRYOBIRDS
Blesbois E, Govoroun M, Hidas A, Liptói K, Pain B, Seigneurin F, Patakiné-Várkonyi E; Barna, J.

1INRA-PRC 37380 Nouzilly France, 2Institute for Small Animal Research 200 Isaszegi road Gödöllö 2100 Hungary, 3U846 INSERM- SBRI, Avenue du Doyen Lépine, Bron, 4SYSAAF 37380 Nouzilly, France

ABSTRACT
The development of new tools for the maintenance of genetic variability in commercial lines and in endangered breeds is very important to improve the adaptation of domestic avian resources to the future challenges of environmental changes and market evolution. CRYOBIRDS is a French-Hungarian project (2010-2014) that aims to develop a large panel of complementary and innovative biotechnologies of reproduction to contribute to the improvement of the management of avian genetic resources. The four goals of the project include: 1) improving the actual methods of storage and use of semen, blastodermal and primordial germ cells; 2) developing new and innovative approaches of use of these cell types and of gonadal tissues; 3) developing quality tests of the cryopreserved cells and tissues to enlarge their use as germ cells resources; 4) creating the Hungarian Avian Germcells Cryobank and increasing the collections of the French Cryobank.
The first results of include a considerable improvement of efficiency of guinea fowl semen cryopreservation with a panel of methods. The development of sperm and blastodermal cells vitrification and of ovarian transfer in endangered chicken breeds is also promising. Advances in cellular reprograming are also obtained. Developments of new quality tests are in due course including DNA markers, transcriptomic and proteomic approach.
This project shares new advances in reproductive biotechnologies and initiates the creation of an international network of avian germcells cryobanks.
KEYWORDS: germinal cells, ovary, sperm, cryopreservation, domestic birds

INTRODUCTION
Recent advances in poultry semen cryopreservation technology have resulted in the emergence of cryobanking, which is now being developed in an increasing number of countries (see Blackburn, 2006; Woelders et al, 2006; Blesbois et al, 2007, Blesbois 2011).These advances have taken into account the understanding of cryobiology applied to animal cells, the basic advances on semen biology, and the specific features of bird reproductive physiology, and they have given rise to methods that must be adapted to each species. Zootechnical adaptations of the use of previously cryopreserved sperm must also be made for each species, strain and line. However, despite these advances, semen freezing is still not accessible to all species and not efficient in low fertility lines.
The cryopreservation of blastodermal cells (BCs) or primordial germ cells (PGCs) followed by their re-implantation in recipient embryos (Nakamura et al., 2010; Mac Donald et al., 2010) or the freezing of ovarian tissues with reimplantation in host females (Song and Silverside, 2007) provide very interesting alternatives to semen freezing. However, these methods are not yet sufficiently effective and too costly for large programs of genetic conservation (Petitte, 2006).
In these conditions, the general goal of the present project is to develop complementary reproductive biotechnologies focused on avian germ cells storage. The specific goal of the project includes in a unique strategy the development and use of the haploid male/female gametes and diploid reproductive cells. This is carried out in 2010-2014 by: I) improving the methods of storage and use of semen and embryonic cells; II) developing new and innovative approaches turned to these cell types and also to gonadal tissues; III) developing quality tests of the cell and tissue cryopreserved to enlarge the capacity of use of these germ cells resources.

I- IMPROVING THE METHODS OF STORAGE AND USE OF SEMEN AND EMBRYONIC CELLS
Semen: in this task, methodology of sperm cryopreservation are studied in chicken semen with different initial quality and are also studied in the guinea fowl where the success of the method is very low due to high sperm plasma membrane rigidity (Blesbois and al., 2005) but the possibility of improvements high (Barna, unpublished data). Very significant improvements of cryopreservation process have been obtained in the guinea fowl by different combinations of cryoprotectants/freezing rate. They are detailed in two papers of the present congress: "Comparison of two cryopreservation protocols of guinea fowl sperm" (Varadi E et al., 2012) and "Improvement of guinea fowl semen cryopreservation" (Seigneurin F, Blesbois E, 2012).
In the chicken, the study of different CPAs still shows the superiority of the CPA Glycerlo Glycerol to conserve the viability of chicken sperm of low quality;
BCs and long term PGCs: In Hungary and in France, the development of freezing methodology and use of these two cell types are in progress. We showed with BCs that the length of storage of the recipient eggs before the incubation has no significant effect on the chimera rate. However, the main limiting factor still remains the cell viability before freezing (with an average of 30% Fig 1) following the cell preparation from the non-incubated embryos. The cryopreservation weakens further the BCs viability after thawing.
Long term PGCs cultures are also in due course and are derived either from the embryonic blood of a 2.5 days old embryo or from the developing gonads of a 6.5 days old embryos. Different media and growth factors are tested and the proliferative resulting cells were assessed for the presence of specific germ cell markers such as AP (alkaline phosphatase) and PAS (Periodic acid Schiff) (Fig2) staining, SSEA1 labelling and expression of DAZL, DDX4 (CVH) germ cell specific genes.

II- DEVELOPING INNOVATIVE NEW APPROACH
In this task, new alternative biotechnologies for the cryopreservation and use of avian reproductive cells are developed. They include i) application of vitrification methods to BCs, PGCs and semen; ii) reprogramming avian embryonic somatic cells in germinal cells; iii) cryopreservation and transfer of gonadal tissues. We already got significant results on BCs and Sperm vitrification and also on gonadal transfer.
Vitrification of BCs and Semen: An efficient support for BCs vitrification and new media of vitrification were developed. They are described in "Preliminary results of vitrification of chicken blastodermal cells with a new type of cryocontainer" (Patakine-Varkonyi et al, 2012) of the present congress.
Semen vitrification is also in progress with the development of mix of cryoprotectants and the use of very rapid freezing rates. The preliminary results show that the same rate of viability post freezing may be obtained with rapid vitrified and classically frozen-thawed semen in the chicken (50% viability) and a lower rate with vitrified guinea fowl semen (30% viability).
Gonadal freezing and transfer: Up to date, this is a very promising method that would give the only opportunity to

and Sperm vitrification and also on gonadal transfer.
Vitrification of BCs and Semen: An efficient support for BCs vitrification and new media of vitrification were developed. They are described in "Preliminary results of vitrification of chicken blastodermal cells with a new type of cryocontainer" (Patakine-Varkonyi et al, 2012) of the present congress.
Semen vitrification is also in progress with the development of mix of cryoprotectants and the use of very rapid freezing rates. The preliminary results show that the same rate of viability post freezing may be obtained with rapid vitrified and classically frozen-thawed semen in the chicken (50% viability) and a lower rate with vitrified guinea fowl semen (30% viability).
Gonadal freezing and transfer: Up to date, this is a very promising method that would give the only opportunity to preserve the female gamete. Our preliminary results are described in "Preliminary results of the application of gonadal tissue transfer in the poultry gene conservation in various chicken breeds" (Liptoi et al., 2012) of the present congress. They show that the breed of the recipient is a limiting factor for the success of the transfer.

III- DEVELOPING QUALITY TESTS OF CELLS AND TISSUES CRYOPRESERVED
The recovery of the full functionality of the reproductive cells and tissues after storage is an absolute requirement. Study of this recovery is often very limited today. In this task, we develop criteria that evaluate a full predictive functionality of the recovered cells. This is done by evaluating the integrity and functionality of different sub cellular fractions that include DNA integrity, expression of gene markers and physiologic cellular function. Specific markers of chimerism will be also developed.
DNA integrity: DNA integrity of fresh and frozen blastodermal cells was measured with two different methods. The RAPD method (Random amplified polymorphic DNA) gives promising results for the future.
Gene expression: the methodology to assess the differential expression of BCs before and after cryopreservation is developed and is presented in "Identification of specific embryonic markers of quality of blastodermal cells used for maintenance of avian genetic diversity" (Govoroun et al., 2012). In parallel, genes markers of pluripotency are studied in "Individual and strain variability of pluripotenty marker gene expression at early chicken embryonic stage (Sztan et al., 2012).
Physiological markers: in this part we evaluate pertinent markers of different cellular physiologic functions altered by cryopreservation. They include markers that will be used on the different types of cells studied, such as peptide markers, and markers more specific of each cell type such acrosomic reaction for sperm. The results presented in "Biomarkers of chicken sperm quality displayed by intact cells MALDI-TOF mass spectrometry" (Labas et al., 2012) show the methodology developed to show differential peptide expression of cells of different quality. The study developed on semen shows a mean of 90 peptides markers differentially expressed between fresh and frozen-thawed sperm.
More specific markers are also developed. Among them, acrosome reaction has been shown to be severely altered by semen freezing and differences have been obtained depending on the cryoprotectant employed (Mocé et al, 2010).

CONCLUSION
The project CRYOBIRS is a bi-national project involving France and Hungary. It shares new advances in the reproductive biotechnologies that may be used for the management of genetic diversity in domestic birds. New advances in technologies that already exist are proposed such as new methods of Guinea fowl semen freezing. New developments are also made in risky solutions such as BCs, PGCs and gonadal transfer to host animals. A large panel of quality evaluations of the reproductive cells is also developed. These evaluations involve research without a priori of specific marker through proteomic approach but also research with candidate markers issued from transcriptomic studies or functional observations.
The future action will consider an amplification of the studies of the biotechnologies and quality developments presented here.
They will also check the feasibility of collecting and freezing biological material at a rather large scale with the new methods set up in the previous tasks.
And finally the construction of the Hungarian bird reproductive cryobank will be built and the French avian reproductive cryobank enriched by the constitution of new collections. This project initiates also the creation of an international network of avian germcells cryobanks.

REFERENCES
Blackurn HD 2006. The national animal germplasm program: challenges and opportunities for poultry genetic resources. Poultry Science 85, 210-215.
Blesbois E, Grasseau I., Seigneurin F. (2005) Membrane fluidity and the ability to survive cryopreservation in domestic bird spermatozoa. Reproduction 129, 371-378.
Blesbois E, Seigneurin F, Grasseau I, Limouzin C, Besnard J, Gourichon D, Coquerelle G, Tixier-Boichard M 2007. Semen cryopreservation for ex-situ management of genetic diversity in chicken. Poultry Science, 87, 555-564.
Blesbois, E. 2011. Freezing Avian Semen. Avian Biology Research 4, 44-50.
Govoroun M, Aubel P, Alves S, Jean C, Blesbois E, Pain B (2012) Identification of specific embryonic markers of quality of blastodermal cells used for maintenance of avian genetic diversity. Proceedings WPC 2012.
Labas V, Harichaux G, Grasseau I, Terlot JD, Alves S, Teixeira AP, Gérard N, Blesbois E (2012) Biomarkers of chicken sperm quality displayed by intact cells MALDI-TOF mass spectrometry. Proceedings WPC 2012.
Liptoi K, Horvath G, Varadi E, Barna J (2012) Preliminary results of the application of gonadal tissue transfer in the poultry gene conservation in various chicken breeds. Proceedings WPC 2012.
Mac donald, J.; Glover, J.D.; Taylor, L.; Sang, H.M.; Mac Grew M. (2010) Characterisation and Germline Transmission of Cultured Avian Primordial Germ Cells. PLos ONE, 5, e15518
Mocé E, Grasseau I, Blesbois E.(2010) Cryoprotectant and Freezing-Process alter the Ability of Chicken Sperm to Acrosome React. Animal Reproduction Science 122, 359-366.
Nakamura, Y.; Usui, F; Miyahara, D.; Mori, T.; Ono, T.; Takeda, K.; Nirasaw, D.; Kagami, H.; Tagami, T. (2010) Efficient system for preservation and regeneration of genetic resources in chicken: concurrent storage of primordial germ celles and live animals from early embryos of a rare indigenous fowl. Reprod. Fertil. Dev., 22, 1237-1246.
Patakine-Varkonyi E, Horvath G, Sztan N, Varadi E, Barna J (2012) Preliminary results of vitrification of chicken blastodermal cells with a new type of cryocontainer. Proceedings WPC 2012.
Petitte, J.N. 2006. Avian germplasm preservation: embryonic stem cells or primordial germ cell. Poultr. Sci. 85, 237-42.
Seigneurin F, Blesbois E (2012) Improvement of guinea fowl semen cryopreservation. Proceedings WPC 2012
Song, Y, and Silversides FG. 2007b. Offspring produced from orthotopic transplantation of chicken ovaries. Poult Sci. 86,107-111.
Sztan N, Hidas A, Govoroun M, Pain B (2012) Individual and strain variability of pluripotenty marker gene expression at early chicken embryonic stage. Proceedings WPC 2012
Varadi E, Vegi B, Liptoi K, Barna J,(2012) Comparison of two cryopreservation protocols of guinea fowl sperm. Proceedings WPC 2012
Woelders, H., Zuidberg, A., Hiemstra, S.J. 2006. Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poultr. Sci. 85:216-222.

Fig 1: Kinetic of viability (measured with Propidium Iodide/Sybr14) of Blastodermal cells after cryopresrvation

Fig2: In vitro long term cultured PGC amplified (A) and positive for PAS reaction (B)

附錄四

20130722

NO

DATE

Talk with Elisabeth

Cryopreserve — Reproductive cells ↑ (Main sperm for birds)

DNA

PGG (Bertrand P. → ES cells)

- regulation
- factors
- female、male

↑ start just six month ago

Cryobanking

Project: Gonad Conservation

be able to use!!!

2 Leaders < Elisabeth Bloeisis
Bertrand Pain

5 scientists

4 technicians

2 pHD students

⇓

Whole Team!

May the joy and happiness around you today and always.

NO
DATE

Cryobirds France-Hungary 2010-2013
Elisabeth. B —— Judit Barna

⇒ Primary Breeders
Improvement of Cryopreservation
- semen
- Blastodermal cells
- PGC culture

- guinea fowl
- turkey
- chicken

✡ Her Suggestions
1. concentration of semen (at least 1/2 Conc as fresh semen)
2.
3. change the tube size
4. check Motility (after 0.5-1 hr)
5. Temperature
6. Glycerol < 2% stepwise dilute 採精後
7. If room T too high, Put diluent before collection = T < 20°C
8 Insemination = notice sperm numbers

NO
DATE

CASA – Esabeth.

- VAP
  VCL
  VSL
  STR X
  LIN X
  ALH X
  BCF X
  Elongation X

  Motility Includes:
  [Progressive, more often
  [Rapid Cells

✡ Correlated
fertility – motility – progressive – rapid

- Motility (CASA, Mass Motility)
  Viability

- Capability of Acrosome
  Acrosome reaction
  PNA Ca2+
  SyBr
  PI
  PNA sperms Green.

May the joy and happiness around you today and always.

附錄五

Genetics Selection Evolution

BioMed Central

Research

Open Access

Performance comparison of dwarf laying hens segregating for the naked neck gene in temperate and subtropical environments

Chih-Feng Chen*[1], David Gourichon[2], Nein-Zu Huang[1], Yen-Pai Lee[1], André Bordas[3] and Michèle Tixier-Boichard[3]

Address: [1]Department of Animal Science, National Chung-Hsing University, 40227 Taichung, Taiwan, [2]INRA, Unité expérimentale PEAT, 37380 Nouzilly, France and [3]INRA, AgroParisTech, UMR1236 Génétique et Diversité Animales, 78350 Jouy-en-Josas, France

Email: Chih-Feng Chen* - cfchen@dragon.nchu.edu.tw; David Gourichon - David.Gourichon@tours.inra.fr; Nein-Zu Huang - hnz.tw@yahoo.com.tw; Yen-Pai Lee - yplee@dragon.nchu.edu.tw; André Bordas - andre.bordas@jouy.inra.fr; Michèle Tixier-Boichard - Michele.Boichard@jouy.inra.fr

* Corresponding author

Published: 16 January 2009

Genetics Selection Evolution 2009, 41:13 doi:10.1186/1297-9686-41-13

Received: 17 December 2008
Accepted: 16 January 2009

This article is available from: http://www.gsejournal.org/content/41/1/13

Abstract

This study compares laying performances between two environments of dwarf laying hen lines segregating for the naked neck mutation (*NA* locus), a selected dwarf line of brown-egg layers and its control line. Layers with one of the three genotypes at the *NA* locus were produced from 11 sires from the control line and 12 sires from the selected line. Two hatches produced 216 adult hens in Taiwan and 297 hens in France. Genetic parameters for laying traits were estimated in each environment and the ranking of sire breeding values was compared between environments. Laying performance was lower, and mortality was higher in Taiwan than in France. The line by environment interaction was highly significant for body weight at 16 weeks, clutch length and egg number, with or without Box-Cox transformation. The selected line was more sensitive to environmental change but in Taiwan it could maintain a higher egg number than the control line. Estimated heritability values in the selected line were higher in France than in Taiwan, but not for all the traits in the control line. The rank correlations between sire breeding values were low within the selected line and slightly higher in the control line. A few sire families showed a good ranking in both environments, suggesting that some families may adapt better to environmental change.

Introduction

In poultry selection programmes, birds are generally raised in a uniform environment to record the selected traits and evaluate genetic values. Thus, most commercial populations are obtained from breeding farms with a controlled environment and are delivered to production farms with variable environments across the world. As a consequence, genotype × environment (GxE) interactions may occur. Under subtropical environments, growth rate as well as egg production are generally depressed by high ambient temperature [1,2]. Introduction of some major genes, such as the naked neck (*NA*) gene, in chicken lines can be used to alleviate heat stress, as discussed in several studies carried out at high ambient temperatures [1,3-5]. Heat tolerance of laying hens is an important issue for the impact of G × E on egg production because the productive period is long and the impact of heat stress increases with the hens' age [6].

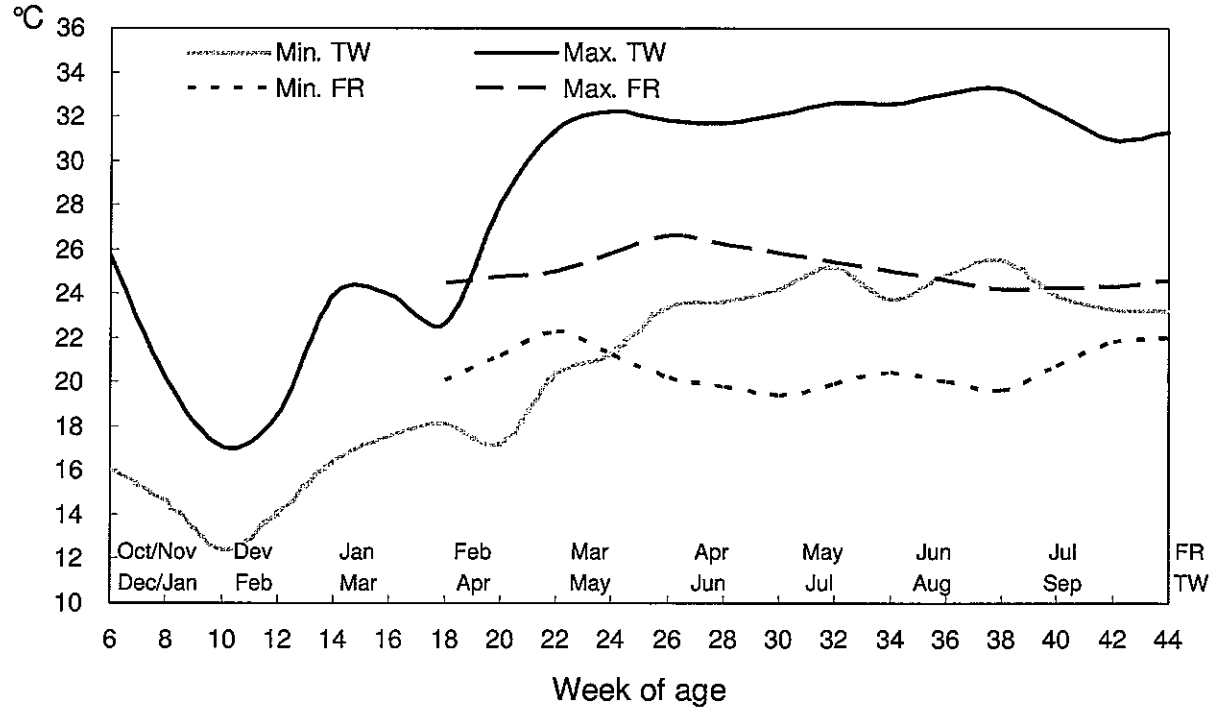


Figure 1
The average maximum and minimum ambient temperature by two weeks in Taiwan (TW) and during the laying period in France (FR). (average temperature, 22 ± 1°C, during the rearing period in France).

normalize trait distribution [13,9]. The parameters (t) were found to be -0.214, and 1.40 for the transformed value of average clutch length (TCL) and for transformed value of total egg number (TEN), respectively.

Statistical analysis

The linear model used for the ANOVA of those traits was as follows:

$$Y_{ijkl} = \mu + E_i + L_j + G_k + (E \times L)_{ij} + (ExG)_{ik} + (L \times G)_{jk} + (E \times L \times G)_{ijk} + e_{ijkl}$$

where Y_{ijkl} = individual observation, μ = overall mean, E_i = fixed effect of environment, i = 1 to 2, L_j = fixed effect of line, j = 1 to 2, G_k = fixed effect of the naked neck genotype, k = 1 to 3, $(E \times L \times G)_{ijk}$ = fixed effect of the third order interaction between all fixed effects, $(E \times L)_{ij}$ = fixed effect of environment-by-line interaction, $(E \times G)_{ik}$ = fixed effect of genotype-by-environment interaction, $(L \times G)_{jk}$ = fixed effect of line-by-genotype interaction, and e_{ijkl} = random error. All analyses were carried with the GLM procedure of the SAS® program library [14]. Means were considered significantly different at $P < 0.05$.

Genetic analysis

Variance and covariance components were estimated using the Restricted Maximum Likelihood (REML) procedure with the VCE package of Groeneveld [15]. The following animal model was applied to all traits on the whole data set:

$$Y_{ij} = \mu + G_i + a_j + e_{ij}$$

where Y_{ij} = individual observation for traits, μ = overall mean, G_i = fixed effect of genotype at the *NA* locus, a_j = random animal effect and e_{ij} = random error. Expectation and variance of the vector of performance, γ, were distributed as follows, in matrix notation:

$$E\begin{bmatrix}\gamma\\ a\\ e\end{bmatrix} = \begin{bmatrix}X\beta\\ 0\\ 0\end{bmatrix} \text{ and } V\begin{bmatrix}a\\ e\end{bmatrix} = \begin{bmatrix}A \otimes G & 0\\ 0 & \bigoplus_{j=1}^{k} R_j\end{bmatrix}$$

where γ = observed performance, a = individual additive genetic value, e = residual, β = vector of genotype fixed effect, X = incidence matrix of vector β, A = numerator relationship matrix; and G = variance-covariance matrix

for the additive genetic effect; k = the number of records, and Rj = residual variance-covariance matrix for animal j. The direct product and direct sum of matrices are indicated by ⊗ and ⊕, respectively.

In order to estimate the genetic correlation between performance measured in the two environments, traits measured in France (F) and Taiwan (T) were treated as distinct $BW16_T$ and $BW16_F$, the subscript indicating the environment. Because a significant environment-by-line interaction was observed for most of the traits, multi-trait analyses were performed separately for each line. It was not possible to analyse all the traits together because of large computing requirements. Therefore eight analyses with four traits each were implemented in the present study. For instance, concerning body weight traits, one four-trait analysis ($BW16_F$, $BW16_T$, ABW_F, ABW_T) was performed in each line.

Sire breeding values were evaluated in each environment for each trait by best linear unbiased prediction (BLUP) using a mixed linear model with the PEST package [16], with the same model described above. A Spearman correlation analysis was used to compare the sire breeding values obtained either in France or in Taiwan.

Results

Total mortality in Taiwan was 22.8% and 23.6% for control and selected lines, respectively, whereas it was below 5% in France. There was no occurrence of epidemic infectious disease in either country. In Taiwan, a hot foehn wind caused a rise in temperature up to 40.3°C during 7 h on July 1st. On that day, mortalities were 6.2%, 13.6% and 5.5% for *NA*N/NA*N*, *NA*NA/NA*N* and *NA*NA/NA*NA* genotypes, respectively. During that week, cumulative mortalities were 7.8%, 14.6% and 9.4% for each of the three genotypes, respectively. Finally, the experiment included 297 hens in France and 216 hens in Taiwan. The proportion of hens carrying the homozygous *NA*NA/NA*NA* genotype was slightly lower in Taiwan (20–22%) as compared to France (25%).

Effects of environment, line, genotype and interactions

The third order interaction between main effects was never significant, although it was at the limit of significance for egg weight ($P < 0.10$). There were no significant interactions between the naked neck genotype and either the line or the environment (Table 1).

The line by environment interaction was highly significant for BW16, clutch length and egg number, with or without Box-Cox transformation (Table 1). Body weight differed between lines in Taiwan to a larger extent than in France: the decrease in body weight observed in Taiwan as compared to France was stronger for the selected line. Clutch length and egg number were smaller in Taiwan and to a larger extent for the selected line *i.e.* the decrease in average clutch length, the selected trait, was 26.6% (3.19 vs. 2.34) and 59.4% (13.89 vs. 5.64) in the control and the selected lines, respectively. However, performance of the selected line still remained much higher than that of the control line in both environments.

The naked neck genotype had a significant effect on body weight at 16 weeks (BW16), age at first egg (AFE) and egg weight (EW) (Table 1). Homozygous naked neck hens had a lower BW16 and a higher AFE, except in the control line in France. They also exhibited a larger EW, but the difference was significant only for the control line in France, and for the selected line in Taiwan (Tables 2 and 3). Thus, the *NA* gene limited the negative impact of the change in environment on egg weight in the selected line.

All traits were significantly influenced by environment and line. For all traits, the performance measured in France was better than the performance obtained in Taiwan. In France, the mean values for CL, EN and LR were respectively 3.19 (untransformed), 120 (untransformed),

Table 1: Results of the analysis of variance for the performance of laying hens according to environment (France or Taiwan) to selection background (selected line or control line) and to the naked neck genotype (homozygous normal, heterozygous or homozygous for the naked neck mutation)

Traits	Environment (E)	Line (L)	Genotype (G)	E × L	G × E	G × L	G × E × L
Body weight at 16 weeks	***	***	*	***			
Age at first egg	***	***	*				
Clutch length	***	***		***			
Clutch length (Trans.)	**	***		***			
Egg number at 44 weeks	***	***		*			
Egg number at 44 weeks (Trans.)	***	***		***			
Laying rate	***	***					
Adult body weight	***	***					
Egg weight	***	*	*				+

Significant at +, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$

Table 2: Performances of egg production in France

Traits (Number of birds)	Control line			Selection line		
	*NA*N/NA*N*[1] (20)	*NA*NA/NA*N* (56)	*NA*NA/NA*NA* (31)	*NA*N/NA*N* (43)	*NA*NA/NA*N* (93)	*NA*NA/NA*NA* (44)
Body weight at 16 weeks, g	1420 ± 37[2]	1435 ± 16	1443 ± 33	1457 ± 21 [a]	1441 ± 18 [a]	1371 ± 18 [b]
Age at first egg, day	139.5 ± 4.0	135.5 ± 1.2	139.4 ± 1.7	130.1 ± 1.2	133.8 ± 2.1	132.8 ± 1.2
Clutch length, egg	2.93 ± 0.24	3.32 ± 0.18	3.11 ± 0.20	13.35 ± 1.76	13.67 ± 0.86	11.93 ± 0.92
Clutch length (Trans.)	6.69 ± 0.50	7.38 ± 0.29	7.04 ± 0.40	14.01 ± 0.32	13.96 ± 0.30	13.62 ± 0.36
Egg number at 44 weeks	111.5 ± 8.1	123.0 ± 3.9	120.1 ± 5.0	167.4 ± 1.6	163.6 ± 2.8	157.8 ± 4.1
Egg number at 44 weeks (Trans.)	81.4 ± 7.1	92.0 ± 3.6	88.9 ± 4.8	139.5 ± 1.9	136.2 ± 2.7	129.5 ± 4.1
Laying rate, %	63.0 ± 4.4	68.9 ± 2.1	68.8 ± 2.7	91.0 ± 0.8	90.3 ± 1.2	87.0 ± 2.1
Adult body weight, g	2156 ± 71	2141 ± 39	2181 ± 67	2071 ± 27 [a]	2004 ± 26 [ab]	1924 ± 36 [b]
Egg weight, g	49.8 ± 1.1 [b]	51.8 ± 0.6 [ab]	52.4 ± 0.8 [a]	51.0 ± 0.6	51.2 ± 0.5	50.3 ± 0.5

[1] *NA*N/NA*N*: normally feathered; *NA*NA/NA*N*: heterozygous naked neck; *NA*NA/NA*NA*: homozygous naked neck
[2] The values are mean ± standard error
[a, b] Different superscripts indicate significant difference within line at a given environment

and 66.9 in the control line and 13.17 (untransformed), 163 (untransformed), and 89.4 in the selected line. In Taiwan, the average clutch length was 2.34 and 5.64 (untransformed values) for the control and selected lines, respectively. After normalization by Box-Cox transformation, the relative decrease of average clutch length in Taiwan was similar for both lines and close to -30% except for the normally feathered hens in the control line, but, in absolute values, the decrease was larger in the higher performing line After normalization, the total egg number in Taiwan as compared to France was even more reduced (by about 40%) as a result of the increase in age at first egg observed in Taiwan (by about 15%). Laying rate was reduced by 20% in Taiwan in both lines.

Comparison of the laying curves obtained in France and Taiwan (Figures 2 and 3) showed differences due to a delayed onset of lay, a lower peak of egg production with a slightly better persistency of lay until the age of 44 weeks. There was a three-week delay of the onset of lay for both lines in Taiwan and, in the selected line, the homozygous *NA*NA/NA*NA* genotype reached the peak of egg production later, but this was not significant in the control line.

Feed consumption was measured between 31 and 34 weeks of age in Taiwan only. There were no significant differences between lines (2163 vs 2267 for control line vs selection line), or among genotypes (2171, 2281, 2219 for *NA*N/NA*N*, *NA*NA/NA*N*, *NA*NA/NA*NA* genotypes).

Genetic parameters

Estimates of heritability and genetic correlations between traits measured in the two environments are shown in Table 4 for each line. Heritability values estimated for the

Table 3: Performances of egg production in Taiwan

Traits (Number of birds)	Control line			Selection line		
	*NA*N/NA*N*[1] (25)	*NA*NA/NA*N* (36)	*NA*NA/NA*NA* (16)	*NA*N/NA*N* (49)	*NA*NA/NA*N* (59)	*NA*NA/NA*NA* (31)
Body weight at 16 weeks, g	1360 ± 38[2,a]	1295 ± 32 [ab]	1261 ± 21 [b]	1213 ± 23 [a]	1213 ± 19 [a]	1143 ± 24[b]
Age at first egg, day	157.6 ± 2.6 [a]	160.7 ± 2.8 [ab]	167.4 ± 3.1 [b]	153.4 ± 2.0	152.2 ± 1.4	157.4 ± 2.6
Clutch length, egg	2.60 ± 0.17	2.27 ± 0.11	2.09 ± 0.15	5.53 ± 0.40	6.50 ± 0.52	5.46 ± 0.62
Clutch length (Trans.)	6.11 ± 0.35	5.30 ± 0.28	4.81 ± 0.44	9.85 ± 0.39	10.34 ± 0.42	9.68 ± 0.51
Egg number at 44 weeks	80.8 ± 5.2	76.8 ± 4.1	76.7 ± 4.9	112.4 ± 3.7	110.9 ± 4.1	107.4 ± 3.5
Egg number at 44 weeks (Trans.)	51.7 ± 4.3	48.2 ± 3.4	47.5 ± 4.1	81.1 ± 3.4	80.3 ± 3.7	75.5 ± 3.4
Laying rate, %	53.7 ± 3.3	52.3 ± 2.6	54.4 ± 3.1	72.6 ± 2.1	71.0 ± 2.5	71.4 ± 2.2
Adult body weight, g	1720 ± 42	1748 ± 41	1677 ± 36	1587 ± 28	1585 ± 25	1551 ± 37
Egg weight, g	48.3 ± 0.5	49.5 ± 0.7	49.6 ± 0.9	46.6 ± 0.4 [b]	47.2 ± 0.5 [b]	49.3 ± 0.6 [a]

[1] *NA*N/NA*N*: normally feathered; *NA*NA/NA*N*: heterozygous naked neck; *NA*NA/NA*NA*: homozygous naked neck
[2] The values are mean ± standard error
[a, b] Different superscripts indicate significant differences within lines at a given environment

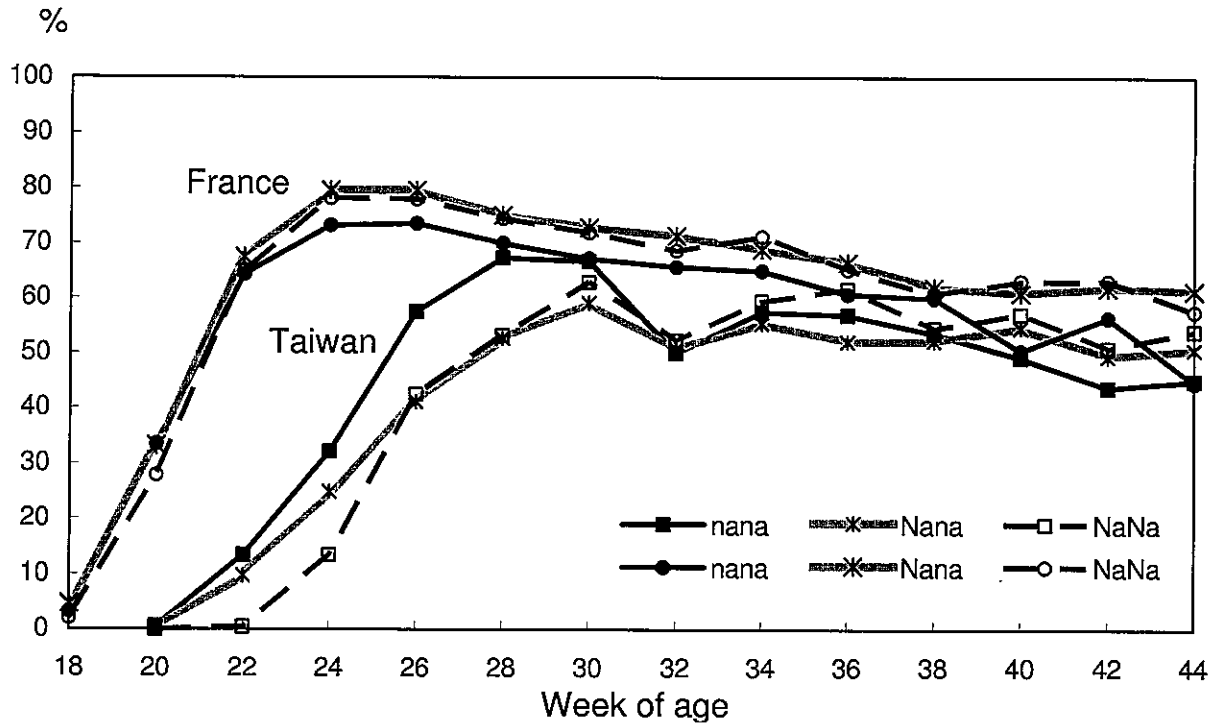


Figure 2
Hen-day laying rate by two weeks for three genotypes of the control line in France and Taiwan.

selected line were generally higher for traits measured in France and the difference was noticeable for body weight, egg number, laying rate and egg weight. Heritability values obtained in Taiwan for the selected line were very low for egg number and laying rate. Since in most cases, the estimated heritability values exhibited larger asymptotic errors for the control line than for the selected line (because of a limited number of animals), the differences in estimates between the two environments were not as remarkable. However, in some cases high values were obtained *i.e.* for normalized clutch length, which showed a higher estimated heritability in Taiwan (0.91) than in France (0.57).

Genetic correlations between traits measured in the two environments for the selected line were high and not different from 1 for body weight and egg weight (from 0.82 to 0.96); values were lower and much less accurate for egg laying traits, so that it may be suggested that most of these correlations were null; only age at first egg exhibited a reasonably low asymptotic error, supporting the hypothesis that the correlation did not differ from zero. A different picture was observed for the control line: the highest correlation was observed for clutch length in both environments and was very close to 1, whereas correlations obtained for egg number, laying rate and body weight ranged from 0.34 to 0.48. Actually, correlations for body weight, egg weight and age at first egg were rather lower than in the selected line and exhibited the largest standard errors of all estimates for the control line, suggesting that they may not differ from zero.

The Spearman rank correlations of sire breeding values estimated between the two environments are listed in Table 5 together with their probability values. Most of these correlations were not significant but some differences between lines may be pointed out. Correlations for egg production traits (clutch length, egg number) were moderate to high in the control line (0.72 to 0.88) and differed significantly from zero, whereas they did not differ from zero in the selected line. On the opposite, correlations for body weight and egg weight were higher in the selected line (0.48 to 0.70) than in the control line; the correlation was significant for egg weight in the selected line but not in the control line. The correlation for age at first egg was low and non significant in both lines.

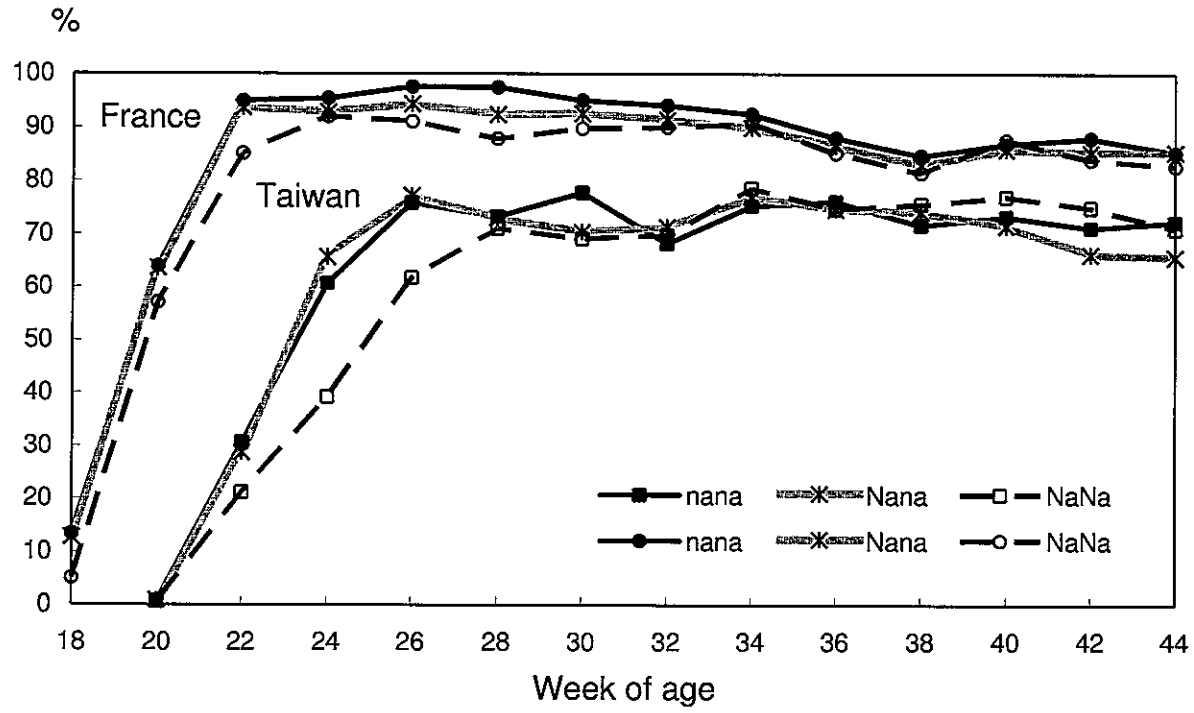


Figure 3
Hen-day laying rate by two weeks for three genotypes of selected lines in France and Taiwan.

Discussion

Effects of environment, line, genotype and interaction

The effect of environment was quite large. The performances of all traits were lower in Taiwan and mortality was very high for both lines. Since there was no outbreak of infectious disease, this high level of mortality is rather difficult to explain. It may be due to the cumulated effect of several environmental and husbandry factors differing between France and Taiwan, which affected lines to a similar extent. For instance, these lines are never debeaked in France. To our knowledge, it has never been reported that debeaking has an effect on feed intake and behaviour in dwarf brown-egg layers. Since hens were housed in indi-

Table 4: Estimated heritability for each trait measured in each environment and genetic correlations considering the records of a given performance in both environments (France-Taiwan) as different traits within each line

Traits	h^2 in France		h^2 in Taiwan		Genetic correlation between traits defined for each measurement in each environment	
	Control line	Selection line	Control line	Selection line	Control line	Selection line
Body weight at 16 weeks	0.51 ± 0.16[1]	0.85 ± 0.15	0.64 ± 0.16	0.46 ± 0.12	0.44 ± 0.25	0.82 ± 0.13
Age at first egg	0.17 ± 0.11	0.49 ± 0.11	0.31 ± 0.14	0.42 ± 0.12	-0.45 ± 0.38	0.16 ± 0.16
Clutch length (trans.)	0.57 ± 0.11	0.20 ± 0.08	0.91 ± 0.13	0.12 ± 0.07	0.98 ± 0.06	0.49 ± 0.45
Egg number at 44 weeks (trans.)	0.49 ± 0.17	0.32 ± 0.12	0.37 ± 0.18	0.01 ± 0.03	0.48 ± 0.09	-0.10 ± 1.48
Laying rate	0.50 ± 0.18	0.27 ± 0.10	0.35 ± 0.16	0.07 ± 0.06	0.23 ± 0.09	0.14 ± 0.58
Adult body weight	0.52 ± 0.14	0.69 ± 0.14	0.38 ± 0.17	0.34 ± 0.13	0.34 ± 0.25	0.96 ± 0.05
Egg weight	0.39 ± 0.09	0.60 ± 0.10	0.18 ± 0.09	0.38 ± 0.10	0.42 ± 0.35	0.86 ± 0.13

[1] asymptotic standard error

Table 5: Spearman correlation coefficients between sire breeding values estimated in both environments

Traits	Control line (sire number = 9)	Selected line (sire number = 12)
Body weight at 16 weeks	0.45, P = 0.224	0.50, P = 0.101
Age at first egg	-0.20, P = 0.606	0.07, P = 0.829
Clutch length (Trans.)	0.88, P = 0.002	0.23, P = 0.471
Egg number at 44 weeks (Trans.)	0.72, P = 0.030	-0.21, P = 0.513
Laying rate	0.57, P = 0.112	-0.01, P = 0.966
Adult body weight	0.12, P = 0.765	0.48, P = 0.112
Egg weight	0.47, P = 0.205	0.70, P = 0.011

vidual cages, debeaking was not expected to influence performance greatly.

A significant interaction between environments and lines was found for laying traits, which indicated that the selected line was more sensitive to environmental changes than the control line for production level. Thus, performance level influenced the adaptation of laying hens. However, it should be noted that, in Taiwan, the selected line maintained a higher performance for laying rate than the control line. Among the main differences between the two environments, ambient temperature, lighting regimen and diet composition are discussed in more detail below.

Concerning ambient temperature, a favourable effect on hens' adaptation was expected with the naked neck gene. Indeed, at a constant ambient temperature of 32°C, the *NA* gene has been shown to limit the negative effect of long-term heat stress on egg production and feed efficiency traits [3], because of its effect on sensible heat loss, which is increased due to lower feather cover [12,17]. In the present study, the average daily minimum and maximum temperatures ranged between 24.1°C to 32.4°C during the laying period. However, there was no significant effect of the naked neck genotype on laying traits and no interaction between naked neck genotype and environment. This suggests that the naturally cycling temperature was not so stressful for laying hens. In broilers, chickens exposed to temperature cycling over a wide range of 24°C - 35°C, exhibit a similar growth rate to that recorded in birds exposed to a constant temperature [18].

Furthermore, the *NA* gene was not expected to influence adaptation to other husbandry factors than temperature, such as lighting or feed composition. Indeed, since all these factors differed between France and Taiwan, the resulting effect of the *NA* gene could not be identical to the effect observed when only the temperature factor was changed. This study suggests that ambient temperature is not the main driving factor of adaptation of layers to subtropical environments in experimental conditions.

Concerning lighting regimen, it has been shown to play a very important role in the onset of sexual maturity. A constant or decreasing amount of daily light will delay sexual maturity in growing birds. Indeed, in the present study, the amount of daily light in Taiwan decreased during the rearing period, which probably explains the three-week delay for the onset of lay observed in both lines. The control line exhibited the highest values for onset of lay, as previously reported [10]. The delay appeared to be longer for the naked neck hens in both lines, which may also be due to the lower body weight observed with this genotype in Taiwan and in France for the selected line only. Chen and Tixier-Boichard [10] have already observed this effect of the naked neck gene on sexual maturity for the same dwarf lines.

Concerning feed composition, the higher protein percentage used in Taiwan is expected to have a positive effect on egg weight [19], but this is not what we observed. Since egg weight is generally strongly correlated to body weight, the negative effect of the subtropical environment on body weight is probably the main explanation for the lower egg weight observed in Taiwan.

Genetic parameters

Changes in environment affected genetic parameters to a larger extent in the selected line than in the control line. In the selected line, the estimated heritabilities exhibited higher values for performance recorded in France than in Taiwan, which could be explained by the large environmental variability in Taiwan. Coefficients of variation for clutch length, egg number and laying rate were much larger in Taiwan than in France for the selected line except for the homozygous naked neck hens; the same trend was observed, with a lower magnitude, in the control line (Table 6). Coefficients of variation were generally smaller and remained more stable between environments for body weight, egg weight and sexual maturity in both lines (Table 6). The heritability values for AFE (0.49), TEN (0.32) and LR (0.27) obtained for the selected line in France were close to estimates previously published for the same population [10]. For the selected trait TCL and

Table 6: Coefficients of variation for traits measured in France and Taiwan, for each line and each genotype at the naked neck locus

Traits	Control line						Selection line					
	NA*N/NA*N[1]		***NA*NA/NA*N***		***NA*NA/ NA*NA***		***NA*N/NA*N***		***NA*NA/NA*N***		***NA*NA/ NA*NA***	
	France	Taiwan	France	Taiwan	France	Taiwan	France	Taiwan	France	Taiwan	France	Taiwan
Body weight at 16 weeks	11.7	14.1	8.19	14.9	12.8	6.74	9.48	13.3	12.0	12.1	8.94	11.5
Age at first egg	12.8	8.16	6.4	10.5	6.8	7.33	5.9	9.3	15.4	7.34	5.8	9.02
Clutch length (Trans.)	33.2	28.8	29.6	32.2	**31.7**	**36.9**	**14.7**	**28.2**	**20.7**	**31.9**	**17.7**	**29.1**
Egg number at 44 weeks (Trans.)	39.1	41.8	**28.9**	**42.2**	30.0	34.4	**8.92**	**29.5**	**18.9**	**36.4**	21.1	25.2
Laying rate	31.4	30.8	**23.1**	**30.3**	22.1	22.7	**5.4**	**20.0**	**12.3**	**27.2**	16.4	16.9
Adult body weight	14.7	11.5	13.6	13.7	17.2	8.51	8.39	12.5	12.6	11.9	12.5	13.4
Egg weight	9.79	5.3	8.17	8.82	8.64	7.39	7.45	6.66	8.79	8.10	6.44	6.84

The largest relative changes between environments are indicated in bold characters.
[1] *NA*N/NA*N*: normally feathered; *NA*NA/NA*N*: heterozygous naked neck; *NA*NA/NA*NA*: homozygous naked neck

traits strongly correlated with it (egg number and laying rate), heritability values were higher in the control line, whatever the environment, which is consistent with the decrease in genetic variance due to past selection. This suggests that selection to improve laying performance in Taiwan may be difficult in the selected line.

Estimating genetic correlations between traits measured in two environments is one approach to reveal within-line genotype × environment (G × E) interactions [20]. Correlations significantly lower than 1 indicate the occurrence of GxE interactions. Our results indicate that G × E interactions are more important for laying traits than for body weight and egg weight in the selected line but absent for clutch length in the control line. This is consistent with

Table 7: Sire breeding values in the selected line, for traits measured in both environments, data are sorted according to the breeding value for clutch length (transformed for normalization) estimated from performance recorded in France

Sire ID	Clutch length (Trans.)		Egg number at 44 weeks (Trans.)		Laying rate		Adult body weight		Egg weight	
	France	Taiwan	France	Taiwan	France	Taiwan	France	Taiwan	France	Taiwan
23	1.3375	-0.6509	9.774	-0.3085	4.26	-1.1137	91.14	-2.08	2.8653	0.2951
19	**1.1622**	**0.4122**	**5.462**	**0.4112**	**4.242**	**1.4648**	**-95.65**	**65.05**	**0.9138**	**-0.0335**
16	**0.4291**	**0.6636**	**1.453**	**0.4402**	**2.64**	**2.681**	**-183.05**	**-86.1**	**-0.5016**	**0.2148**
21	0.4168	-0.6866	9.636	-0.4325	2.453	-2.4114	-28.82	-57.71	1.269	0.5487
12	0.3184	0.6349	-17.522	0.258	-2.653	2.0701	244.85	72.69	5.6541	2.8713
17	0.2211	0.1083	8.155	0.3474	2.263	0.2681	-69.44	-16.66	-5.4449	-2.4796
18	-0.025	0.3935	7.5	0.2238	1.128	0.3332	9.24	38.79	0.0707	-0.7562
15	-0.127	-0.3903	-4.79	-0.2068	-0.565	0.9742	-222.19	-9.06	-2.2545	1.3863
22	-0.2054	0.121	5.669	-0.3079	1.886	-2.2254	2.83	-96.1	-2.3982	-3.7489
13	-1.0237	0.4648	-0.058	0.2109	-1.338	1.1587	143.12	75.12	-2.6192	-0.8452
14	-1.1715	0.0366	-22.879	0.2269	-13.281	0.1669	103.45	-21.82	3.8258	1.5172
20	-1.3385	-1.1083	-2.386	-0.8631	-1.053	-3.3693	4.54	37.88	-1.3587	1.0144

the fact that the highest performing line was more susceptible to environmental change. Concerning age at first egg, very low correlations were observed in both lines, which were similarly affected by the difference in lighting regimen.

Rank correlations between sire breeding values, calculated for 9 and 12 sires from control and selected lines respectively show no correlation for egg production traits in the selected line, and positive but moderate correlations for body weight and egg weight. The situation is almost opposite in the control line, with a significantly positive correlation for egg production traits and low to moderate correlations for body weight and egg weight. Similarly, rank correlation results confirm genetic correlations results. This emphasizes the high impact of GxE interaction on the within-line selection process that should not be ignored in a breeding program. Within the selected line, a few sires (sire 19 and 16) showed a good ranking in both environments for the selected trait, clutch length (Table 7) suggesting that it may be possible to identify some families with a better adaptation capacity to environmental changes.

Conclusion

Breeders regularly mention the importance of GxE interactions within commercial lines, but data obtained for similar families in contrasted environments, as shown here, are generally not published. Use of a control line, derived from the same base population as the selected line, clearly shows that the sensitivity to a new environment, particularly subtropical climate, is increased by past selection. This greater sensitivity is associated with a decrease in heritability of selected traits. Regular testing of a given line across various environments, and integrating such data in the evaluation programme could monitor this impact. Considering the current predictions on future climate changes, this result shows that the components of adaptation to real subtropical conditions need to be better identified in order to anticipate the negative consequences on the climate change. Indeed, the heat tolerance described for naked neck layers in experimental conditions was not found to particularly improve adaptation to the naked neck layers in Taiwan. Identification of a few sire families capable of achieving a good performance in both environments, temperate and subtropical, suggests that selection for improved adaptation capacity may be feasible. However, a large genetic base will be necessary to identify a sufficiently large number of sires to start a selection programme. Other differences between selected lines, not included in the present study, may also have an influence and should be investigated in the future.

Authors' contributions

CFC designed the study, wrote the paper, imported animals, and performed the statistical analysis. DG and NZH supervised, organized and carried out the data collection. YPL and AB participated in the design of the experiment, commented on drafts of the paper. MTB participated in the coordination and design of the study, exported animals, discussed the interpretation of the results and commented on drafts of the paper.

Acknowledgements

The current study is part of a scientific collaboration programme between INRA-NCHU and was supported by INRA and the Council of Agriculture (92AS-1.4.1-ID-17, 93AS-1.4.1-ID-17). Animal caretakers at the experimental unit of INRA, Nouzilly, and at the experimental farm of NCHU, Taichung, are gratefully acknowledged.

References

1. Bordas A, Mérat P: **Effects of the naked-neck gene on traits associated with egg laying in dwarf stock at two temperatures.** *Br Poult Sci* 1984, **25**:195-207.
2. Deeb N, Cahaner A: **Genotype-by-environment interaction with broiler genotypes differing in growth rate, 1. The effects of high ambient temperature and necked-neck genotype on lines differing in genetic background.** *Poult Sci* 2001, **80**:695-702.
3. Chen CF, Bordas A, Gourichon D, Tixier-Boichard M: **Effect of high ambient temperature and naked neck genotype on performance of dwarf brown-egg layers selected for improved clutch length.** *Br Poult Sci* 2004, **45**:346-354.
4. Deeb N, Cahaner A: **The effects of the naked neck genotypes, ambient temperature, and feeding status and their interactions on body temperature and performance of broilers.** *Poult Sci* 1999, **78**:1341-1346.
5. Horst P, Rauen HW, Khoo TH: **Significance of the naked neck gene (Na gene) in poultry breeding in the tropics.** *Proceedings of the 7th European Poultry Conference: 1986; Paris, France* 1986:24-28.
6. Bordas A, Mérat P: **Egg production performances of hens of the NaNa (homozygous naked neck), Nana+ (heterozygous) and na+na+ (normal plumage) genotype from a brown-egg dwarf (dw) line submitted to high constant temperature or to high temperature with periodic fluctuations.** *Arch Geflugelk* 1992, **56**:22-27.
7. Mérat P: **Potential usefulness of the Na (Naked Neck) gene in poultry production.** *World's Poult Sci J* 1986, **42**:124-142.
8. Mérat P: **The sex-linked dwarf gene in the broiler chicken industry.** *World's Poult Sci J* 1984, **40**:10-18.
9. Chen CF, Tixier-Boichard M: **Estimation of genetic variability and selection response for clutch length in dwarf brown-egg layers carrying or not naked neck gene.** *Genet Sel Evol* 2003, **35**:219-238.
10. Chen CF, Tixier-Boichard M: **Correlated response to long-term selection for clutch length in dwarf brown-egg layers carrying or not the naked neck gene.** *Poult Sci* 2003, **83**:709-720.
11. Tixier-Boichard M, Boitard M, Coquerelle G, Mérat P: **Genetic improvement of clutch length in dwarf brown-egg layers: additional selection response with the naked neck gene.** *Proceedings of the 20th World's Poultry Congress, 2–5 September 1996; New Delhi, India* 1996, **1**:453-458.
12. Bordas A, Mérat P, Sergent D, Ricard FH: **Influence of the Na (naked neck) gene on growth, feed consumption and body composition of chicken according to environmental temperature.** *Ann Génét Sél Anim* 1978, **10**:209-231.
13. Besbes B, Ducrocq V, Foulley JL, Protais M, Tavernier A, Tixier-Boichard M, Beaumont C: **Box-Cox transformation of egg-production traits of laying hens to improve genetic parameter estimation and breeding evaluation.** *Livest Prod Sci* 1993, **33**:313-326.
14. SAS® Institute Inc: *SAS/STAT® User's Guide, Version 6.12. Cary, NC, USA* 1999.

At INRA, a selection experiment was undertaken to improve egg production of brown-egg layers by increasing clutch length, in the presence of two major genes known to improve heat tolerance, the naked neck gene [7] and the sex-linked dwarf gene [8]. After 16 generations, significant genetic progress on egg production was observed in two dwarf lines, one carrying the *NA* gene and one not carrying it [9-11]. The reduction of feather mass due to the *NA* gene is lower in heterozygous birds than in homozygous birds, *i.e.* 27% and 22% respectively for heterozygous females and males, and 41% and 33% respectively for homozygous females and males [12]. Heat tolerance is significantly improved by the presence of the *NA* gene and significant genotype by temperature interactions were observed for egg production, egg mass and feed intake under a 32°C constant temperature environment, which represents a severe temperature stress [3]. The purpose of this paper was to study the laying performance in real subtropical conditions of the genotypes obtained from the selection experiment on clutch length, in comparison to the performance in the selection environment. In order to investigate adaptation of hens, genetic parameters for laying traits were estimated in each environment and the ranking of sire breeding values was compared between environments.

Methods

Genetic background

Two lines of dwarf brown-egg layers established from a common base population in 1985 have been selected on average clutch length for 16 generations. The selection procedure has been described by Chen and Tixier-Boichard [9]. One line (L1) was normally feathered and the other (L2) was homozygous for the *NA*NA* mutation; a control line segregating for the *NA*NA* mutation was also maintained. In 2001, both selected lines were crossed to produce a new line segregating for the *NA*NA* mutation, which was maintained with a mild selection pressure on average clutch length. In 2003, 11 males and 44 females from the control line, and 12 males and 48 females from the newly formed selected line, were used to generate individuals with the three possible genotypes at the *NA* locus *i.e.* normally feathered (*NA*N/NA*N*), heterozygous carriers of the naked neck mutation (*NA*NA/NA*N*) or homozygous carriers (*NA*NA/NA*NA*). Chicks were obtained in two hatches at a three-week interval for rearing in Taiwan and France, respectively.

Husbandry

Taiwan

On November 28, 2003, one-day-old chicks were imported to Taiwan. After a 10-day quarantine, they were transferred to the experimental farm of the National Chung-Hsing University. All the chicks were debeaked and reared in floor pens up to 16 weeks. Chicks were vaccinated against the following diseases Marek's disease, fowl pox, Newcastle disease, infectious bronchitis, infectious bursal disease, respiratory enteritic orphan, infectious laryngo-tracheitis, infectious coryza, avian encephalomyelitis and egg drop syndrome. At 17 weeks of age, pullets were housed in individual cages in open house, supplied with ground water and reared in natural daylight. The length of daylight increased gradually from 10.5 to 12 h between ages 4 and 16 weeks and then from 17 weeks of age till the end of the test, a fixed light regimen 14L:10D was applied. This lighting regimen had been used throughout the selection experiment in France and was taken as the reference lighting regimen for the laying period. Water and food were supplied *ad libitum*, layer mash contained 18.0% CP and 11.5 MJ ME/kg and temperature and relative humidity were monitored continuously. Figure 1 shows the curve of daily maximum and minimum temperatures in the chicken house.

France

On September 18, 2003, full-sibs were reared in the experimental farm of INRA, Tours. All chicks were reared in floor pens up to 16 weeks. Chicks were vaccinated against the following diseases: Marek's disease, infectious bronchitis, Gumboro, Newcastle disease and avian encephalomyelitis. At 17 weeks of age, pullets were housed in individual cages in a windowless house with light regimens fixed as 10L:14D and 14L:10D in growth and laying periods, respectively. This lighting regimen had been used since several years for experimental lines in France and was taken as the reference lighting regimen for the laying period. Water and food were supplied *ad libitum*. Monthly averages of maximal and minimal temperatures were 25 ± 1°C and 21 ± 1°C, respectively (February to July/18 to 44 weeks of age). Relative humidity ranged from 60 to 80%. Layer mash contained 16.4% CP and 11.2 MJ ME/kg.

Variables under study

The egg number (EN) was recorded from the age at first egg (AFE) to 44 weeks of age. For each hen, the laying rate (LR) was obtained from the ratio of egg number (whatever the egg status) to the number of days since the first egg. A break of one day at least between ovipositions was taken as the end of a clutch. The average clutch length (CL) was calculated as the arithmetic mean of all clutches recorded, from the first egg until 44 weeks of age. In addition, egg weight (EW) was obtained at 34 weeks of age (36 weeks in France), by collecting two eggs per hen laid on consecutive days within a week. The body weights were measured at the entry in the poultry house (BW16) and at adult age (ABW) *i.e.* 34 weeks in Taiwan, and 40 weeks in France.

In order to satisfy the classical hypothesis for describing traits with polygenic inheritance via a linear model with normal error, Box-Cox power transformation was used to